REVIEW

Gangliosides and Atherosclerosis

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The ganglioside levels in atherosclerotic lesions of human aorta are considerably higher than those in unaffected areas of aorta, and atherosclerotic patients frequently have increased concentrations of serum gangliosides. The present review summarizes recent findings that suggest the possible involvement of aortic gangliosides in platelet activation and adhesion of platelets to the vessel wall. The effect of gangliosides on the structure of low density lipoproteins (LDL), on the interaction of LDL with macrophages and hepatic cells and on the LDL-regulated biosynthesis of cholesterol is also discussed. *In vitro* experiments have demonstrated that a major ganglioside of the intima of atherosclerotic aorta induces rapid adhesion, aggregation and spreading of platelets. Moreover, gangliosides present in elevated amounts in the intercellular space of atherosclerotic aortic tissue modify the surface structure and stimulate aggregation of LDL. Ganglioside-modified LDL are readily recognized and taken up by macrophages, while preincubation of LDL with low concentrations of gangliosides inhibits LDL binding to hepatic cells. Thus, ganglioside enrichment of LDL is likely to interfere with LDL clearance *via* the hepatic cells. Thus, ganglioside enrichment of LDL is likely to interfere with LDL clearance *via* the hepatic LDL receptor, and to stimulate binding of LDL to the scavenger receptor of macrophages. It is postulated that high ganglioside levels in the aorta and serum may be an additional risk factor in atherosclerosis.
***Lipids* 29, 1–5 (1994).**

In the development of atherosclerosis, the aortic wall is damaged, and platelets, monocytes and low density lipoproteins (LDL) arrive at the site of injury and contribute to the formation of atherosclerotic plaques that eventually lead to thrombosis. In 1975, Breckenridge *et al.* (1) reported that the ganglioside content of the aorta increased dramatically during atherosclerosis. Although gangliosides were subsequently implicated in cell adhesion (2) and LDL-cell interactions (3,4), Breckenridge *et al.*'s finding attracted little attention, and today only sparse data on the involvement of gangliosides in the events leading to atherosclerosis are available. We summarize the results of some recent studies aimed at clarifying the effect of gangliosides on the structure and function of LDL, and on platelet adhesion, plaque formation and atherogenesis.

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Abbreviations: ApoB, apolipoprotein B; GSL, glycosphingolipids; LDL, low density lipoproteins; MAH, mouse ascites hepatoma; WHHL rabbits, Watanabe hereditable hyperlipidemic rabbits.

The abbreviations used for gangliosides are standard abbreviations as recommended by Svennerholm [Svennerholm, L. (1964) *J. Lipid Res. 5*, 145–155].

Ganglioside changes in aortic tissue related to atherosclerosis. According to Breckenridge *et al.* (2), the level of total gangliosides (lipid-bound sialic acid) in healthy aorta is low, but increases progressively in aortas containing fatty streaks, plaques and ulcerated lesions. These studies were performed on whole aorta tissue. In order to obtain additional information, we studied the glycosphingolipids (GSL) of intima and of underlying areas of aortas taken from subjects who had died after myocardial infarction (5–7).

Our data showed important differences between the GSL profiles of the intima and the underlying areas, as well as between cells taken from affected and unaffected areas of the aorta. In unaffected areas a major difference between the intima and the underlying media was the absence in intima of glucosyl and lactosylceramide, whereas in the underlying media these substances accounted for at least one-third of the neutral GSL (6). The main ganglioside in both intima and media was GM3; however, in contrast to the media, the intima proved to contain relatively high amounts of GD3 (5). A similar characteristic ganglioside pattern was detected in the aorta of Watanabe hereditable hyperlipidemic (WHHL) rabbits, an animal model for human familial hypercholestrolemia (8). In the aorta of these rabbits, the level of total gangliosides increases 12-fold in comparison to that in normal rabbit aorta. One of the most predominant gangliosides in the aorta of WHHL rabbits (next to GM3) is GD3, which, in normal aortas, is almost undetectable. While GM3 is the major ganglioside in all normal extraneural tissues, GD3 is considered a characteristic antigen of many fast-growing, undifferentiated or malignant cells (see Ref. 9 for a review). It has been suspected for a long time that there might be similarities between tumor growth and the fast proliferation of smooth muscle cells during the development of atherosclerosis (10). During recent years a considerable body of evidence has accumulated suggesting a special role of gangliosides in tumorigenesis (11). Some facts supporting this theory include the effect of gangliosides on the loss of contact inhibition in tumor cells (11), the enhancement of tumor formation by gangliosides produced by highly tumorigenic cells in weakly tumorigenic cells (12), the suppression of natural immunity by gangliosides shed by tumor cells (13,14) and the high expression in tumor cells of certain gangliosides, such as GD3 and GD2, which are little or not at all expressed in normal extraneural tissues (reviewed in Ref. 9). Thus, the comparatively high level of GD3 in the intima of atherosclerotic aorta supports the idea that some analogy exists between certain steps in atherogenesis and in tumorigenesis.

It is noteworthy that in whole tissues of the atherosclerotic aorta, the ganglioside concentration is considerably higher than in isolated cells, suggesting that even higher amounts of gangliosides may accumulate in the intercellular space (7). This again is reminiscent of the situation with many tumor cells that intensively shed

their gangliosides, thus creating high ganglioside concentrations within their microenvironments (reviewed in Ref. 9).

In this context, it is interesting to note that antibodies raised to intimal cells have been found to be directed against gangliosides (15). This prompted us to study the role of gangliosides in the adhesion of platelets to the vessel wall. To this end we investigated the interaction of human platelets with individual aortic gangliosides adsorbed to plastic (16). In these experiments only GD3, but no other ganglioside, dramatically stimulated platelet adhesion, spreading and aggregation (Fig. 1). Taken together, this suggests that intimal GD3 exposed to the lumen after endothelial injury may be a factor responsible for platelet attachment to the damaged vessel wall.

The binding of gangliosides to LDL. A high LDL concentration in serum is a major risk factor for atherosclerosis, and it is now firmly established that the cholesterol that accumulates in atherosclerotic lesions originates primarily from LDL. Incidentally, LDLs are also the main carriers of plasma gangliosides (17), but until recently little was known about the effect of gangliosides on LDL structure and function. In order to fill this gap, we undertook an investigation of the binding of gangliosides to LDL and of the changes in LDL structure brought about by their interaction with gangliosides present in human plasma (18,19).

The ganglioside content of serum has been studied intensively. For normal serum most authors reported average values in the order of 10 nmol/mL of serum (17,20–24), and LDL isolated from normal plasma contain only a few ganglioside molecules per lipoprotein particle (17,24). In hypercholesterolemia, however, the serum ganglioside level was found to be considerably higher (25).

We found that even larger amounts of gangliosides can associate with lipoproteins upon incubation of isolated LDL with exogenous gangliosides (19). Such ganglioside-rich LDL may be postulated to form *in vivo* in situations where lipoproteins exist in a relatively high-ganglioside environment, for example, in the local microenvironment of stimulated macrophages, which are known to actively release gangliosides, particularly GD3 (26). In the concentration range of 20–120 μM, the ganglioside uptake by LDL proved to be proportional to the ganglioside concentration without any trend toward saturation. Washing with salt solutions removed only a minor portion of the gangliosides bound, indicating that nonelectrostatic forces seem to play an important role in the formation of LDL-ganglioside adducts.

Nevertheless, a small yet saturable binding component, due to specific interaction of ganglioside with certain sites on LDL apolipoprotein B (apoB), also appears to exist. This was demonstrated in a study on the binding of gangliosides to LDL that was immobilized on Sepharose (18). The binding of mono- and disialogangliosides to Sepharose-immobilized LDL was saturable and depended on the specific ganglioside structure (18). Ganglioside binding to the LDL-affinity gel could be reversed by washing the gel with dilute salt solutions. Mild trypsinization of the ganglioside-loaded LDL-affinity gel also removed most of the gangliosides.

In radioligand assays of LDL, the extent of specific binding can be evaluated by adding excess heparin to the incubation medium. The amount of LDL that binds to cells under such conditions corresponds to nonspecific interaction. When excess heparin was added to the LDL-affinity gel simultaneously with gangliosides, binding of the latter was substantially reduced (18).

Thus, while the uptake of gangliosides by native LDL particles (*i.e.*, LDL isolated from plasma by standard

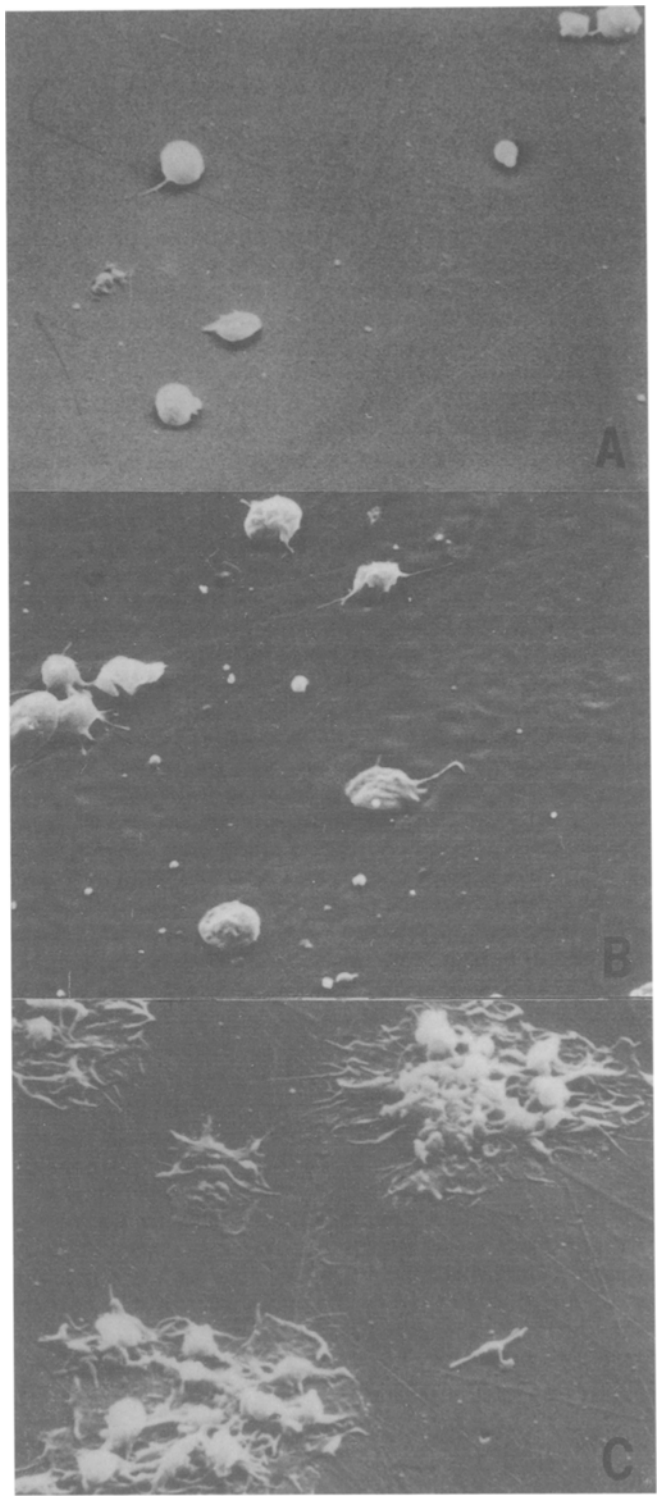

FIG. 1. Interaction of platelets with gangliosides coated on plastic. A, no ganglioside; B, GM1, C, GD3 (Ref. 16).

methods) is predominantly nonspecific and hydrophobic, the binding of gangliosides to immobilized LDL appears to be largely hydrophilic and more specific.

Gangliosides modify LDL structure. Even small amounts of gangliosides are able to induce a molecular reorganization of the LDL surface. This can be deduced from the fact that ganglioside binding to LDL is accompanied by changes in the intrinsic fluorescence of apoB which contains more than 30 tryptophan residues (19). Upon incubation with gangliosides, the apoB fluorescence maximum is shifted to longer wavelengths indicating that some of the tryptophyls are translocated to more polar environments (19). On the other hand, the apoB tryptophyls become, on the average, less accessible to quenching by aqueous iodide. Thus gangliosides appear to cause certain conformational changes of apoB molecules on the LDL surface. These changes, in turn, alter the molecular packing of the LDL surface phospholipids that surround apoB. This conclusion is based on the observation that small amounts of gangliosides had a pronounced effect on the fluorescence anisotropy of LDL labeled with fluorescent phospholipids in the outer monolayer (Fig. 2) (18). Interestingly, the mobility of the probes and, hence, the fluidity of their microenvironment, changed in different directions depending on the carbohydrate structure of the ganglioside. The effect was saturated at concentrations corresponding to less than 10 ganglioside molecules per LDL particle. As a result of the ganglioside-induced changes of the LDL surface, the LDL particles slowly aggregate, forming small adducts containing an average of three native LDL particles (19).

Involvement of gangliosides in LDL-cell interactions. The removal of LDL from the plasma is achieved by two alternate pathways. The main pathway is mediated by specific LDL receptors that are present on the surface of most mammalian cells (27). However, some cell types lacking the "normal" LDL receptors are able to take up modified forms of LDL, recognizing them by so-called "scavenger" receptors. Typical representatives of cells expressing the normal LDL receptor are fibroblasts and hepatocytes, whereas monocytes (macrophages), Kupffer cells and endothelial cells are characterized by the presence of scavenger receptors (for a recent review, see Ref. 28). Binding of LDL to the normal receptor is followed by internalization of the lipoprotein particle, and this initiates a chain of events, including the inhibition of cholesterol synthesis, the stimulation of cholesterol esterification and the inhibition of LDL-receptor synthesis (29).

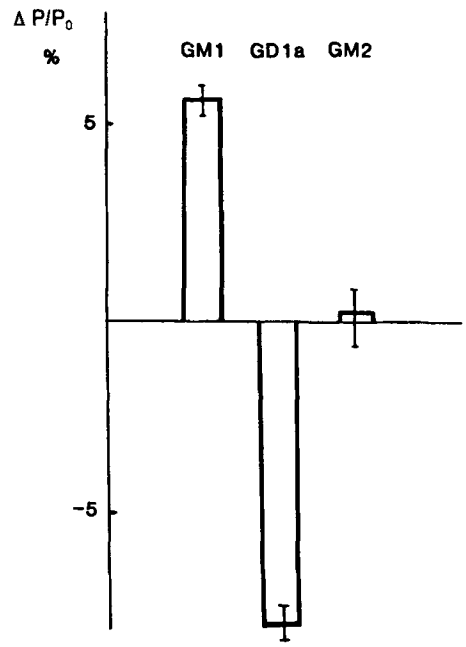

FIG. 2. Ganglioside-induced changes in the fluorescence polarization of low density lipoprotein (LDL) labeled in the outer monolayer by a fluorescent analogue of sphingomyelin (Ref. 18). Ganglioside concentration, 10^{-9} M; LDL concentration, 1 mg protein/mL. $\Delta P = P - P_0$ where P_0 and P are the fluorescence polarization values before and after addition of ganglioside, respectively.

Studies on the effect of gangliosides and other sialoglycoconjugates on LDL-cell interactions have led to apparently conflicting results. According to some investigators, the receptor-mediated uptake of LDL increases after enzymatic desialylation of the lipoproteins (3,30; see also Ref. 31). On the other hand, it has been noted by some authors that increasing the number of sialic acid residues on the surface of recipient cells stimulates receptor-mediated LDL uptake (32), whereas others found that the uptake increased after partial desialylation of the cells (33). These differences may be related to the use of different recipient cells with different ganglioside profiles and different LDL receptor types. In our studies we therefore employed two cell types of well-known ganglioside composition expressing different types of LDL receptors as LDL recipients, namely mouse ascites hepatoma (MAH) cells having the high affinity receptor for native human LDL (18) and mouse resident peritoneal macrophages characterized by the presence of scavenger receptors. In the liver cholesterol, synthesis is under feedback control. In many hepatomas this control mechanism is impaired or lost (34); however, we found that in MAH cells, human native LDL inhibited cholesterol synthesis in a manner similar to liver cells (18).

To elucidate the role of cell-surface gangliosides in the interaction of these cells with LDL, we compared the binding of LDL to native MAH cells, neuraminidase-treated cells and neuraminidase-treated cells subsequently loaded with gangliosides. The results revealed that LDL binding correlated positively with the number of sialic acid residues on the cell surface, whereas preincubation of LDL with gangliosides inhibited LDL binding to MAH cells (18). Such behavior may be explained by assuming the existence on the LDL surface of binding sites recognizing accessible sialic acid residues. Upon incubation with exogenously added gangliosides, these sites are blocked. Conversely, during preincubation of neuraminidase-treated cells with gangliosides, part of the latter insert their lipophilic ceramide moieties into the outer leaf of the plasma membrane thus increasing the number of LDL binding sites on the cell surface. As shown in Figure 3, neuraminidase treatment of the cells abolished the inhibitory effect of LDL on cholesterol biosynthesis, and this effect could be restored by ganglioside loading of the neuraminidase-treated cells (18). Taking into account that MAH cells still retain many features of normal hepatocytes, it seems probable that exogenous gangliosides could affect binding of LDL to the liver in a similar way, thus modulating the main pathway of LDL removal from the bloodstream.

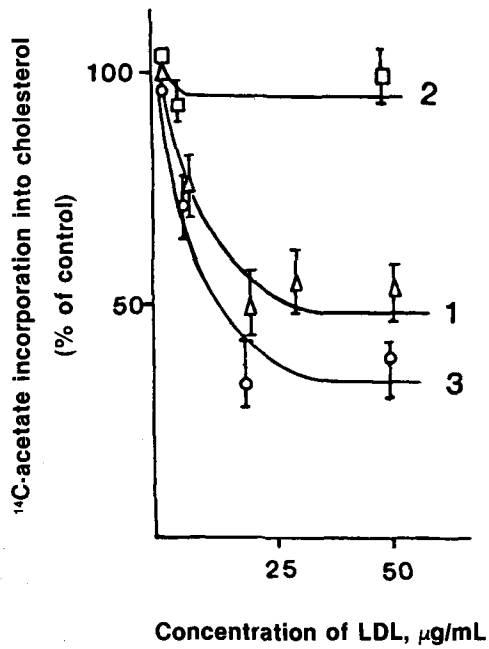

FIG. 3. Effect of low density lipoprotein (LDL) and gangliosides on cholesterol biosynthesis in mouse ascites hepatoma cells (Ref. 18). 1, Native cells; 2, neuraminidase-treated cells; and 3, neuraminidase-treated cells subsequently loaded with GDIa.

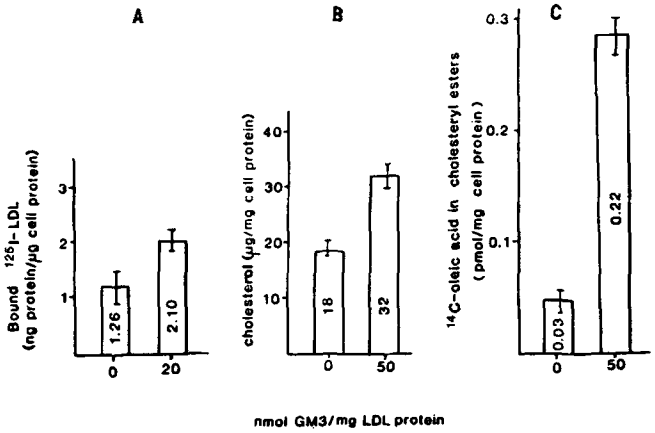

FIG. 4. Effect of gangliosides on the interaction of low density lipoproteins (LDL) with mouse peritoneal macrophages (Ref. 35). A, [^{125}I]LDL uptake (ng LDL protein/μg cell protein); B, cholesterol accumulation (ng cholesterol/μg cell protein); C, cholesteryl ester biosynthesis (pmol of [^{14}C]oleate/mg cell protein); 10^6 cells were incubated with 100 μg LDL protein or LDL preincubated wth 5 nmol of ganglioside GM3.

As mentioned above, monocytes/macrophages are another cell type playing a key role in the pathogenesis of atherosclerosis. One of the earliest events in atherogenesis is the migration of monocytes from blood into the arterial wall where they differentiate into macrophages and accumulate high amounts of cholesteryl esters and other lipids. These lipid-loaded cells are called foam cells. The lipid that accumulates in foam cells is believed to be derived from LDL, in spite of the fact that native LDL are poorly recognized by macrophages *in vitro* and that even high amounts of native LDL, when incubated with macrophages, do not induce accumulation of much cholesteryl ester within the cells (29). It has been shown, however, that several *in vitro* modifications of LDL can transform them into particles which are rapidly taken up by macrophages *via* the scavenger receptor and can cause them to accumulate cholesterol and to transform into foam cells (reviewed in Ref. 28). It is therefore believed that some type of LDL modification that transforms LDL into a form recognized by the scavenger receptor on macrophages occurs *in vivo* in the arterial intima. One possibility is LDL peroxidation induced by monocytes and endothelial cells; however, the exact nature of the *in vivo* LDL modification is as yet unknown. As a possible candidate we suggest the changes in LDL structure brought about by interaction of the lipoprotein with gangliosides. As shown in Figure 4, addition of small amounts of ganglioside markedly increased the uptake of LDL by mouse peritoneal macrophages (35). For still unknown reasons, with native LDL, the uptake by macrophages was relatively intense; nevertheless pretreatment of the LDL with GM3 resulted in a twofold increase in LDL uptake and accumulation of cholesterol (Figs. 4a and b). Moreover, the stimulating effect of GM3-treated LDL on the synthesis of cholesteryl esters in macrophages was at least seven times higher than in the presence of native LDL (Fig. 4c, Ref. 35). A similar enhancement was seen in regard to the accumulation of triglycerides (not shown). These data indicate that in ganglioside-rich, damaged regions of the aorta, LDL ganglioside complexes may be formed, which aggregate and are taken up by phagocytes residing within the lesions, eventually giving rise to foam cells.

The exact nature of the ganglioside-induced modifications of LDL that allows scavenger receptor binding remains unknown. One possibility is neutralization of the lysine ε-aminogroups of apoB by the negatively charged gangliosides. Similar charge neutralizations have been suggested to be the result of various chemical modifications converting native LDL into species recognized by the scavenger receptor (29,36-38). Charge neutralization may also be the reason for the enhancement of LDL aggregation induced by gangliosides (19). However, it was recently demonstrated that LDL uptake by macrophages requires neither charge neutralization nor aggregation (39). Thus, the understanding of the ganglioside-induced changes ultimately responsible for LDL uptake by macrophages will require additional studies, particularly of the competition between ganglioside-modified LDL and other modified forms of the lipoprotein.

Taken together, the evidence for involvement of gangliosides in atherogenesis still remains fragmentary. However, the coincidence of many facts, such as the relatively high ganglioside level in sera of atherosclerotic patients and in atherosclerotic aortas, the ability of intimal gangliosides to induce platelet adhesion, spreading and aggregation, the effects of gangliosides on receptor-mediated LDL-cell interactions, the modification of LDL by gangliosides and the ability of macrophages to recognize ganglioside-modified LDL, undoubtedly deserves further investigation.

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ARTICLE

Regulation of Rat Liver Microsomal Cholesterol Ester Hydrolase by Reversible Phosphorylation

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The regulation of neutral cholesterol ester hydrolase activity by changes in its phosphorylation state was studied in rat liver microsomes. Treatment with cAMP-dependent protein kinase resulted in increased enzyme activity, which was further enhanced by the addition of cAMP and MgATP. Consistent activations were also achieved with $MgCl_2$ and MgATP, the magnesium effect being abolished by ethylenediaminetetraacetic acid and adenosine triphosphate. Cholesterol ester hydrolase was activated twofold by free calcium and Ca^{2+}/calmodulin; this latter effect was blocked by the chelator ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid and the calmodulin antagonist trifluoperazine. The phosphatase inhibitors pyrophosphate and glycerophosphate led to marked and dose-dependent increases in esterase activity, whereas okadaic acid elicited no effect. Furthermore, pyrophosphate and okadaic acid did not change the increases in enzyme activity promoted by Ca^{2+}, Ca^{2+}/calmodulin, Mg^{2+} and MgATP. Cholesterol ester hydrolase was inactivated in a concentration-dependent manner by nonspecific alkaline phosphatases. In cAMP-dependent protein kinase/cAMP- or Ca^{2+}/calmodulin-activated microsomes, a time-dependent loss of activation in cholesteryl oleate hydrolysis was caused by alkaline phosphatase. These findings suggest that microsomal cholesterol ester hydrolase is activated through cAMP and Ca^{2+}/calmodulin phosphorylation, whereas enzyme deactivation is dependent on phosphatase action.
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Free and esterified cholesterol in liver are subject to constant turnover. Acyl-CoA:cholesterol acyltransferase (ACAT) esterifies excess intracellular cholesterol with long chain fatty acids (1), and the resulting cholesteryl esters are utilized for bile and lipoprotein synthesis or stored in cytosolic lipid droplets and in the membranes of the endoplasmic reticulum (2). Mobilization of these esters, when required, is brought about by the catalytic action of the neutral cholesterol ester hydrolases (CEH) located in the cytosol and in microsomes (3,4). Soluble cholesterol esterase has been purified and rather well characterized (5,6); however, few studies have been reported on the regulation and function of the microsomal enzyme. Rat liver neutral microsomal CEH has been partially solubilized (7), and 75% of its activity was found on the luminal side of the rough endoplasmic reticulum (8). The enzyme displays a circadian rhythm unrelated to the feeding status (9). Contradictory reports have appeared with respect to the existence of compensatory changes in microsomal CEH activity in rat (10) and hamster (11) in response to altered cholesterol flux to the liver. The activity of neutral microsomal CEH was found to be different from that of hormone-sensitive lipase (12), neutral retinyl ester hydrolase and acid CEH, also located in microsomes (13). It is thought that microsomal CEH may be involved in controlling the partitioning of cholesteryl esters toward the bile canaliculi or the blood stream and in maintaining the balance between free and esterified cholesterol in endoplasmic reticulum. Moreover, CEH may hydrolyze esterified cholesterol stored in the cytosol (14), although the cytosolic CEH is believed to be mainly responsible for this process (15,16).

Reversible phosphorylation is one of the most remarkable mechanisms involved in the complex regulation of cholesterol metabolism. The regulatory role of reversible phosphorylation is fairly well documented for 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (17–19), whereas controversial results have been reported for cholesterol 7α-hydroxylase (20–23) and ACAT (24–27). Many studies have demonstrated that cytosolic CEH can be activated by cAMP-dependent phosphorylation in steroidogenic and other nonhepatic tissues (28–33). Whether liver-soluble CEH is regulated by covalent modification remains unresolved. Evidence for activation of cytosolic CEH by cAMP- and Ca^{2+}-mediated phosphorylation has been presented in rat liver (34), while evidence suggesting inactivation by phosphorylation was presented in hepatocyte suspensions (35,36).

To our knowledge, it is not known what effect, if any, cellular protein kinases and phosphoprotein phosphatases have on the liver microsomal CEH reaction. The present study is the first attempt to address this question. A cell-free system and several agents that are widely used to promote either protein phosphorylation or dephosphorylation have been utilized. The results presented here offer considerable evidence (although indirect) that neutral microsomal CEH may be activated through cAMP- and Ca^{2+}/calmodulin (CaM)-mediated mechanisms, whereas enzyme inhibition is dependent on phosphatase action.

MATERIALS AND METHODS

Chemicals. Cholesteryl [1-^{14}C]oleate (52 mCi/mmol) and [1-^{14}C]oleic acid (57.4 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, United Kingdom). Bovine serum albumin (essentially fatty acid free), cholesteryl oleate, sodium taurocholate, adenosine triphosphate (ATP), cAMP, okadaic acid, trifluoperazine, calmodulin, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid (EGTA), pyrophosphate, glycerophosphate, cAMP-dependent protein kinase (Type I), and bovine kidney (Type XXXI), chicken intestine (Type V) and *Escherichia coli* (Type III) alkaline phosphatases were from Sigma Chemical Co. (St. Louis, MO). One unit of calmodulin will stimulate 0.016 activated units of phosphodiesterase,

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ATP, adenosine triphosphate; CaM, calmodulin; CEH, cholesterol ester hydrolase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA.

3′:5′-cyclic nucleotide, to 50% of the maximum activity of the enzyme when saturated with activator, in the presence of 0.01 mM Ca^{2+}, pH 7.5, at 30°C. One unit of alkaline phosphatase is defined as the amount that hydrolyzes 1 μmol of *p*-nitrophenyl phosphate per minute in a 0.1 M glycine buffer, pH 10.4, containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, at 37°C. Egg yolk lecithin was from Lipid Products (Nutfield, United Kingdom). All other chemicals were reagent grade.

Preparation of rat liver microsomes. Female Sprague-Dawley rats (180–200 g) were kept at alternating 12-h light/dark periods (lights on from 0700 to 1900) and used at 0900. Rats were given free access to drinking water and a pelleted rat chow low in fat (A04 Panlab; Panlab, Barcelona, Spain). Animals were anesthetized by exposure to diethyl ether vapors and killed by exsanguination. The livers were immediately removed, rinsed in ice-cold saline solution, blotted dry, weighed and homogenized in 4 vol of 20 mM Tris-HCl buffer, pH 7.4, containing 200 mM sucrose, 10 mM EDTA and 50 mM NaF. Microsomal membranes were prepared by differential centrifugation (1,000 × *g*, 15 min; 22,000 × *g*, 20 min; 105,000 × *g*, 60 min), washed once with the homogenization buffer and resuspended in 1 vol of 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose. The entire fractionation was done at 4°C. Microsomes were stored at −20°C under nitrogen. CEH activity remained essentially unchanged at this temperature for at least seven days.

Assay of CEH activity. Substrate was prepared as mixed micelles of cholesteryl oleate, lecithin and sodium taurocholate in a 1:4:2 molar proportion basically as described by Hajjar *et al.* (37). In pilot experiments, assay conditions for CEH activity were optimized for time, protein amount and substrate concentration. The enzyme activity was linear up to 75 min of incubation and from 0.1 to 1 mg protein, and showed typical Michaelis-Menten kinetics with an apparent Km of around 90 μM. The assay mixture typically contained, in a total volume of 0.4 mL, 40 nmol cholesteryl oleate (6.25 mCi/mmol), 0.4 mg microsomal protein and 100 mM imidazole-HCl buffer, pH 6.8. The mixture was preincubated at 37°C for the time and with the agents as indicated in the legends to the figures (shown later) prior to the addition of micellar substrate to initiate the reaction. The incubation was run for 45 min and was stopped by the addition of 5 mL chloroform/methanol/*n*-heptane (1.25:1.4:1, by vol) containing unlabeled cholesteryl oleate as carrier and 1 mL of 50 mM sodium carbonate/50 mM sodium borate buffer, pH 11. The labeled free fatty acids released were measured by liquid scintillation counting in aliquots of the upper phase. The partitioning of free fatty acids was monitored by the addition of known amounts of [1-^{14}C]oleic acid to blank assays with routine recovery of 85%. CEH activity was expressed as Units/mg protein, one Unit corresponding to the hydrolysis of one pmol cholesteryl oleate per minute at 37°C.

Chemical and statistical analysis. Protein was determined as described by Bradford (38), using bovine serum albumin as standard. The unpaired Student's *t*-test was used to determine significance.

RESULTS

Activation of neutral microsomal CEH by phosphorylating agents. To induce CEH phosphorylation, microsomes were treated with commercial cAMP-dependent protein kinase or with compounds which selectively activate the various protein kinases associated with the microsomal preparation. As seen in Table 1, cAMP-dependent protein kinase produced a marked increase in CEH activity, which was further enhanced by cAMP and MgATP. This stimulation was slightly reduced in the presence of uncomplexed ATP (Table 1), whereas ATP alone (0.5–5 mM) had no effect on CEH activity (data not shown). Treatment of microsomes with $MgCl_2$ or MgATP also resulted in concentration-dependent activation (Fig. 1).

TABLE 1

Effect of cAMP-Dependent Protein Kinase (PK), cAMP, Adenosine Triphosphate (ATP) and MgATP on Neutral Microsomal Cholesterol Ester Hydrolase (CEH) Activity[a]

Additions	CEH (relative activity, %)
None	100 ± 3
PK, 50 μg	124 ± 3[b]
PK, 100 μg	137 ± 5[b]
PK, 100 μg + cAMP, 100 μM	158 ± 3[b]
PK, 100 μg + cAMP, 1 mM	196 ± 18[b]
PK, 100 μg + cAMP, 100 μM + MgATP, 2 mM	179 ± 6[b]
PK, 100 μg + cAMP, 100 μM + ATP, 2 mM	126 ± 5[b]

[a]Microsomes were preincubated for 10 min in the presence of the specified compounds before assaying CEH activity, as described in the Materials and Methods section. The absolute value of 100% activity was 39.2 ± 1.2 Units/mg protein. The values are the mean ± SE of three experiments done in duplicate.
[b]$P \leq 0.005$ *vs.* incubations without additions.

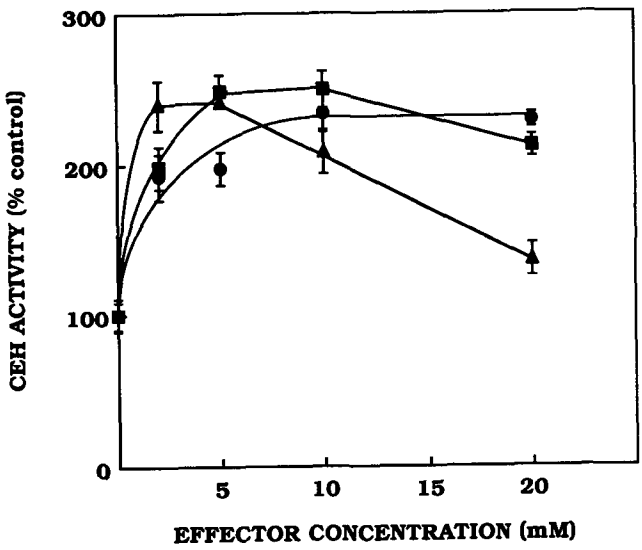

FIG. 1. Activation of neutral microsomal cholesterol ester hydrolase (CEH) by $MgCl_2$ and MgATP. Microsomes were preincubated for 20 min in the presence of $MgCl_2$ (●) or MgATP (■) (1:1, molar ratio) before starting the reaction as described in the Materials and Methods section. In (▲), 5 mg microsomal protein was preincubated for 60 min with $MgCl_2$, then microsomes were re-isolated (105,000 × *g* for 60 min) and assayed for CEH activity. The absolute value of 100% activity was 49.7 ± 3.9 Units/mg protein in microsomes without re-isolation, and 31.7 ± 1.6 in re-isolated microsomes. Each value represents the mean ± SE of three experiments done in duplicate. Vertical bars not shown lie within symbols.

The Mg^{2+} activation was not dependent on the presence of the cation in the enzyme assay mixture, inasmuch as activation of CEH in microsomes re-isolated after preincubation with $MgCl_2$ for 60 min was similar to that observed when the cation was not removed (Fig. 1). An altered sedimentation of the membranes induced by the above physiological salt concentration (39) could account for the decrease in activity found with 20 mM $MgCl_2$. In microsomes re-isolated after activation with $MgCl_2$ (control, 31.7 ± 1.6; 5 mM $MgCl_2$-treated, 72.9 ± 3.8 Units/mg protein), CEH was also enhanced by the addition of 100 μg cAMP-dependent protein kinase and 100 μM cAMP (up to 89.5 ± 6.2 Units/mg protein). These data strongly suggest that $MgCl_2$ promotes a persistent modification of the enzyme, which is reflected in a positive change in activity and which is independent of the cAMP signal transduction system.

The effects of $CaCl_2$ and the Ca^{2+}-binding protein CaM on microsomal CEH activity were studied next. As seen in Table 2, $CaCl_2$ increased CEH activity in a concentration-dependent manner. An even greater enhancement of activity was achieved by addition of CaM, whereas CaM in the absence of exogenous calcium had no effect. The Ca^{2+}/CaM-induced activation was completely blocked by EGTA and by the CaM antagonist trifluoperazine (Fig. 2). The latter compound by itself produced a notable decrease of activity in untreated microsomes. It is evident that both Ca^{2+} and CaM are necessary for maximal enzyme activation and that calcium may also activate CEH activity directly. Stimulation of CEH by the metal ions Mg^{2+} and Ca^{2+} was reversed by ATP and selectively inhibited by the chelators EDTA and EGTA (Table 3). The Ca^{2+} activation of CEH was specifically blocked by EGTA, whereas the Mg^{2+}- and the MgATP-enhanced CEH activity was markedly decreased by EDTA. Mg^{2+} and Ca^{2+}/CaM together produced a greater stimulatory effect than either agent did individually (Table 3). The Mg^{2+} effect seems to dominate, as EGTA did not suppress activation but EDTA did.

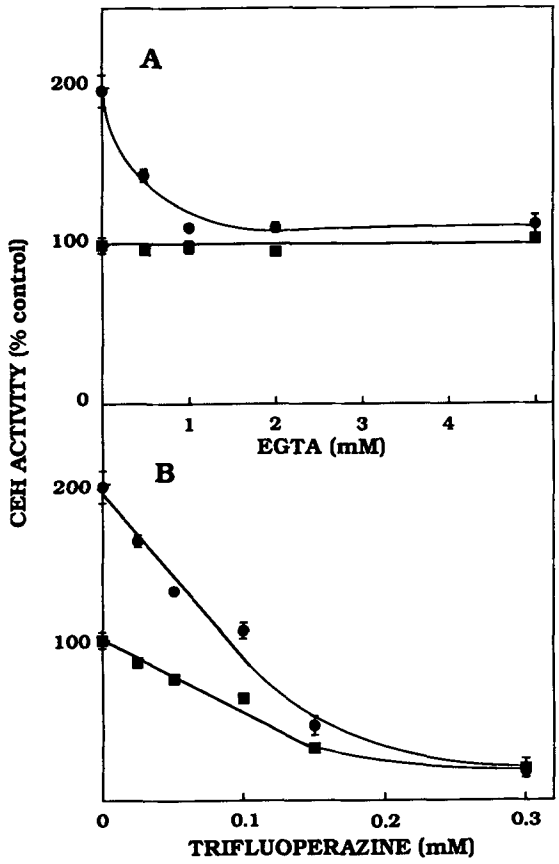

FIG. 2. Effects of ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) and trifluoperazine on the Ca^{2+}/calmodulin-induced stimulation of neutral microsomal cholesterol ester hydrolase (CEH) activity. Microsomes were preincubated for 5 min without (■) or with 1 mM $CaCl_2$/10 Units CaM(●), and increasing concentrations of EGTA (A) or trifluoperazine (B) were added before starting the enzyme reaction. The absolute value of 100% activity was 47.3 ± 2.6 Units/mg protein. Each value represents the mean ± SE of triplicate determinations from a representative experiment of a total of three performed. Vertical bars not shown lie within symbols.

TABLE 2

Effect of Ca^{2+} and Ca^{2+}/Calmodulin on Neutral Microsomal CEH Activity[a]

Additions	CEH (relative activity, %)
None	100 ± 3
$CaCl_2$, 1 mM	137 ± 2[b]
$CaCl_2$, 2 mM	153 ± 8[b]
$CaCl_2$, 5 mM	165 ± 6[b]
Calmodulin, 10 Units	114 ± 9
Calmodulin, 100 Units	102 ± 6
$CaCl_2$, 1 mM + Calmodulin, 10 Units	201 ± 7[b]
$CaCl_2$, 2 mM + Calmodulin, 100 Units	234 ± 9[b]
$CaCl_2$, 2 mM + Calmodulin, 200 Units	258 ± 8[b]

[a]Microsomes were preincubated for 10 min in the presence of the specified compounds before assaying cholesterol ester hydrolase (CEH) activity, as described in the Materials and Methods section. The absolute value of 100% activity was 48.5 ± 1.8 Units/mg protein. The values are the mean ± SE of three experiments done in duplicate.

[b]$P \leq 0.005$ *vs.* incubations without additions.

To determine whether the activating effect of Mg^{2+}, MgATP, Ca^{2+} and Ca^{2+}/CaM might depend on an endogenous phosphatase-related dephosphorylation, CEH activity was measured in the presence of the nonspecific phosphatase inhibitor pyrophosphate or of the types 1 and 2A phosphatase inhibitor okadaic acid (40,41), in microsomes treated with Mg^{2+}, MgATP, Ca^{2+} or Ca^{2+}/CaM (Table 4). Two significant results were obtained. First, pyrophosphate increased neutral CEH activity in control microsomes about 40%. Second, Mg^{2+}, MgATP, Ca^{2+} and Ca^{2+}/CaM activated CEH in the presence of the phosphatase inhibitors.

Inactivation of neutral microsomal CEH by dephosphorylating agents. Treatment of microsomes with nonspecific alkaline phosphoprotein phosphatases from bovine kidney, chicken intestine and *E. coli* resulted in marked concentration-dependent decreases in activity, though the extent of inhibition varied (Fig. 3). Addition of five Units of phosphatase inhibited CEH activity by 50–100%, whereas maximum inhibition was produced by relatively low amounts of *E. coli* phosphatase. A correlation between enzyme activity and the inhibition of

TABLE 3

Effect of ATP, EDTA, EGTA on Neutral CEH Activity in Mg^{2+}-, MgATP-, Ca^{2+}- and Mg^{2+}/ca^{2+}/CaM-Treated Microsomes[a]

Additions	CEH activity (Units/mg protein)			
	Mg^{2+}-treated	MgATP-treated	Ca^{2+}-treated	Mg^{2+}/Ca^{2+}/CaM-treated
None	74.5 ± 3.0	87.3 ± 3.1	78.9 ± 2.9	109.7 ± 6.2
ATP, 2 mM	74.2 ± 3.8	—	66.6 ± 2.1[b]	—
ATP, 5 mM	65.6 ± 2.7[b]	—	52.9 ± 2.4[b]	—
ATP, 10 mM	53.9 ± 2.2[b]	—	46.7 ± 1.8[b]	—
EDTA, 5 mM	51.8 ± 4.5[b]	55.3 ± 1.1[b]	75.2 ± 3.7	63.8 ± 2.7[b]
EGTA, 2 mM	70.3 ± 1.8	91.6 ± 2.9	49.7 ± 2.3[b]	99.7 ± 4.8

[a]Microsomes were preincubated for 10 min with 2 mM $MgCl_2$ or 2 mM MgATP or 2 mM $CaCl_2$ or with 2 mM $MgCl_2$ plus 1 mM $CaCl_2$ and 10 Units of CaM. Then ATP, EDTA or EGTA was added before measuring CEH activity, as described in the Materials and Methods section. Enzyme activity in untreated microsomes was 48.1 ± 2.0 Units/mg protein. The values are the mean ± SE of three experiments done in duplicate. Abbreviations as in Table 1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; CaM, calmodulin.
[b]At least $P < 0.05$ *vs.* activity without additions.

endogenous phosphatases was demonstrated in microsomes treated with the phosphatase inhibitors pyrophosphate and glycerophosphate (Table 5). Concentration-dependent increases in CEH activity were observed with both compounds, although glycerophosphate was less effective than pyrophosphate. The stimulation by pyrophosphate persisted during the incubation, although the effect diminished with time (92, 86, 77 and 69% of increase at 15, 30, 45 and 60 min of incubation, respectively; data not shown). The pyrophosphate-induced activation was slightly enhanced by 5 mM ATP (10%) and 2 mM $MgCl_2$ (17%), and attenuated (34%) by 1 Unit of chicken intestine phosphatase (data not shown). NaF, the phosphatase inhibitor most widely employed, progressively inhibited cholesteryl oleate hydrolysis, even in the presence of the activators, cAMP-dependent protein kinase, Mg^{2+} or Ca^{2+}/CaM (data not shown). The inhibition was found to be reversible, inasmuch as dilution or removal of fluoride from the medium resulted in full recovery of CEH activity (data not shown). Thus, 50 mM NaF was added to the homogenization buffer to avoid spontaneous changes in CEH activity during microsome isolation, but it was excluded from the buffers used for resuspension and the enzyme assay.

These findings suggest a role for endogenous serine/threonine phosphatases in the inactivation of this CEH. Further support for this view is provided by the observation that treatment of microsomes with 1–100 μM orthovanadate, a tyrosine phosphatase inhibitor, did not modify CEH activity (data not shown).

Reversibility of the changes induced by phosphorylation of neutral microsomal CEH. The existence of an activation/deactivation cycle for CEH was investigated using cAMP-dependent protein kinase/cAMP or Ca^{2+}/CaM as activators, and the nonspecific alkaline phosphatase from chicken intestine as an inhibitor. As depicted in

TABLE 4

Effect of Pyrophosphate and Okadaic Acid on Neutral CEH Activity in Mg^{2+}-, MgATP-, Ca^{2+}- and Ca^{2+}/CaM-Treated Microsomes[a]

Microsomal treatment	CEH activity (Units/mg protein)		
	None	Pyrophosphate	Okadaic acid
None	50.0 ± 1.9	70.7 ± 1.1	52.6 ± 2.4
$MgCl_2$	88.5 ± 1.6	90.2 ± 3.4	81.4 ± 1.5
MgATP	81.1 ± 3.7	—	82.7 ± 3.8
$CaCl_2$	73.7 ± 2.4	92.3 ± 7.7	75.6 ± 1.7
$CaCl_2$/CaM	83.4 ± 5.8	87.5 ± 2.6	85.8 ± 1.1

[a]Microsomes were preincubated for 20 min with 2 mM $MgCl_2$, 2 mM MgATP, 1 mM $CaCl_2$ or 1 mM $CaCl_2$ and 10 Units CaM before adding 50 mM pyrophosphate or 1 μM okadaic acid and assaying CEH activity, as described in the Materials and Methods section. Okadaic acid was dissolved in 10% dimethyl sulfoxide (final volume 0.5% of total), and an equivalent amount of the solvent was added to control incubations. The values are the mean ± SE of three experiments done in duplicate. Abbreviations as in Table 3.

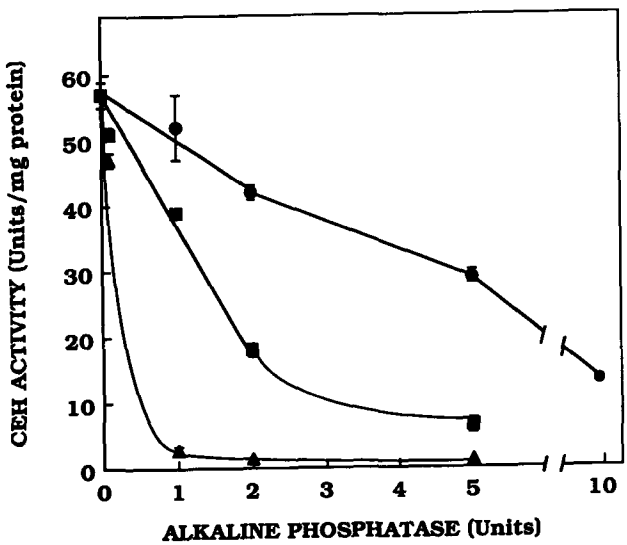

FIG. 3. Concentration-dependent inactivation of neutral microsomal cholesterol ester hydrolase (CEH) by alkaline phosphoprotein phosphatases. Microsomes were preincubated for 20 min with increasing amounts of *Escherichia coli* (▲), chicken intestine (■) and bovine kidney (●) alkaline phosphatases before assaying CEH activity as described in the Materials and Methods section. Each value represents the mean ± SE from three experiments done in duplicate. Vertical bars not shown lie within symbols.

TABLE 5

Effect of Pyrophosphate and Glycerophosphate on Neutral Microsomal CEH Activity[a]

Phosphatase inhibitor (mM)	CEH (relative activity, %)
Pyrophosphate	
5	122 ± 2[b]
10	144 ± 4[b]
20	163 ± 6[b]
50	202 ± 8[b]
Glycerophosphate	
5	106 ± 2
10	110 ± 3
20	133 ± 5[b]
50	172 ± 6[b]

[a]Microsomes were preincubated for 20 min with the specified amount of pyrophosphate or glycerophosphate before assaying cholesterol ester hydrolase (CEH) activity, as described in the Materials and Methods section. The absolute value of 100% activity was 44.8 ± 1.9 Units/mg protein. The values are the mean ± SE of triplicate determinations from a representative experiment of a total of three performed.
[b]At least $P \leq 0.05$ *vs.* activity without additions.

Figure 4, phosphatase counteracted the cAMP-dependent protein kinase/cAMP- and Ca^{2+}/CaM-promoted activations in a similar order of potency, reducing CEH activity almost to the control value after 60 min incubation. A similar experimental design was followed to ascertain whether treatment of deactivated esterase with Ca^{2+}/CaM or cAMP-dependent protein kinase/cAMP would reactivate the enzyme. The phosphatase-induced decrease in CEH activity was not modified by any of the treatments (Fig. 4), even when (at 15 min incubation) phosphatase was blocked by 10 mM pyrophosphate or when 2 mM MgATP was also added (data not shown). However, the addition of 100 μg cAMP-dependent protein kinase and 100 μM cAMP, together with 1 Unit phosphatase, to the microsomal suspension not only counteracted the decrease in CEH but raised its activity *ca.* 80% above the control value (control, 13.4 ± 0.2; phosphatase, 11.3 ± 0.8; phosphatase/cAMP-dependent protein kinase/cAMP, 24.0 ± 1.7 Units/mg protein).

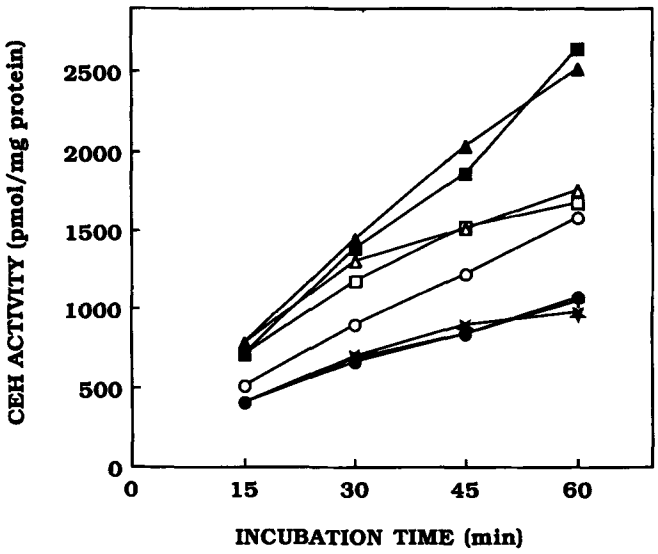

FIG. 4. Time-dependent activation/deactivation of neutral microsomal cholesterol ester hydrolase (CEH) activity. Microsomes were assayed for CEH activity as a function of time after the following treatments: a) none (○); b) 100 μg cAMP-dependent protein kinase/100 μg cAMP (▲); c) 1 mM $CaCl_2$/10 Units CaM (■); and d) 1 Unit chicken intestine phosphatase (●). After 15 min incubation, 1 Unit alkaline phosphatase was added to b (△) and c (□), and 100 μg cAMP-dependent protein kinase/100 μM cAMP (★) or 1 mM $CaCl_2$/10 Units CaM (⊕) were added to d. Points represent the mean values from three experiments.

DISCUSSION

Our results are the first reported evidence that rat liver microsomal CEH might belong to the group of enzymes controlled by reversible phosphorylation. In particular, we provide evidence that *in vitro* conditions favoring phosphorylation in microsomal preparations enhance the rate of cholesteryl oleate hydrolysis, whereas marked decreases in CEH are correlated with the presence of phosphatase activity.

Liver microsomes are known to contain several protein kinases, among them one dependent on cAMP and one dependent on Ca^{2+}/CaM, in addition to phosphoprotein phosphatases and substrates for both classes of enzymes (42). Our findings are consistent with the view that when a microsomal or exogenous cAMP-dependent protein kinase cascade is triggered, CEH activity is enhanced. The potential involvement of a Ca^{2+}/CaM-dependent protein phosphorylation in the activation of CEH is also suggested.

A significant aspect of this work is that the metal ions Mg^{2+} and Ca^{2+} induce consistent increases in CEH activity, which seem to be independent of protein kinases and differ in their response to chelators. Modification of the surface charge of the microsomal membranes by the cations might account for these effects. However, the effect of Ca^{2+} could also be mediated by endogenous CaM bound to the microsomal membranes. This view is supported by the finding that CEH activity in untreated microsomes was inhibited by the CaM antagonist trifluoperazine. These results are in apparent disagreement with those of Coleman and Haynes (43), who reported that rat liver microsomal CEH is not affected by Ca^{2+} and Mg^{2+}. Discrepancies could be due to the way in which the substrate was presented, namely as lipid dispersions in acetone and on the addition of phosphatidylcholine/phosphatidylserine (1:1, w/w) to the incubation medium. The latter addition could alter the interaction between enzyme and cholesteryl oleate which, in turn, may mask further effects of the cations.

Inhibition of CEH by commercial nonspecific phosphatases suggests that microsomal cholesterol esterase is deactivated under conditions at which other proteins are dephosphorylated. An unexpected finding was that the low CEH activity displayed by phosphatase-treated microsomes could not be restored by either cAMP-dependent protein kinase or Ca^{2+}/CaM, whereas the inactivation was prevented when the activators and phosphatase were added to microsomes simultaneously. This suggests that some minimal phosphorylation of the enzyme might be required for activation or, alternatively, that dephosphorylation destabilizes or irreversibly inactivates the enzyme.

If CEH were, indeed, regulated by a reversible phosphorylation mechanism *in vivo*, as suggested by these studies, then endogenous phosphatases should inhibit CEH activity. In fact, CEH was activated and its deactivation prevented by the nonspecific serine/threonine phosphatase inhibitors, pyrophosphate and glycerophosphate. This is the first evidence for involvement of endogenous phosphatases in microsomal CEH inactivation. Serine/threonine phosphatases are classified by their Mg^{2+}, Ca^{2+} and Mn^{2+} dependence and their sensitivity to various inhibitors (40,41). Type 1, 2A, 2B and 2C phosphatases, which account for virtually all detectable phosphatase activities in liver extracts, are directed toward many phosphoproteins, including those controlling cholesterol and fatty acid synthesis (40,41,44-46). Type 1 is the major phosphatase associated with liver endoplasmic reticulum (70%) and glycogen (30%) (40,47). Okadaic acid is particularly useful for distinguishing phosphatases, as it has an especially high affinity for the catalytic subunits of type 1 and 2A phosphatases (41,48). Because the endogenous inhibitor acting on CEH is insensitive to okadaic acid, it is not likely to be a type 1 or 2A phosphatase. Nevertheless, proof of this hypothesis will require systematic studies with purified phosphatases.

Our experiments confirm and extend the idea that a single short-term regulatory mechanism, protein phosphorylation, operates on intracellular cholesterol homeostasis through different mediators. Stimuli shifting phosphorylation through the cAMP or the Ca^{2+}/CaM protein kinase cascade would enhance cholesteryl ester mobilization to supply cholesterol and fatty acids, the former being used for bile acid synthesis (20-23). In contrast, the availability of substrates for cholesteryl ester synthesis is reduced by the AMP-activated protein kinase, which inactivates HMG-CoA reductase and acetyl-CoA carboxylase and antagonizes the activation of the hormone-sensitive lipase by cAMP-dependent protein kinase (49,50). Whether liver ACAT is regulated by protein phosphorylation remains to be determined (24-27). Inasmuch as the present scheme for a coordinated regulation of cholesterol metabolism envisions the liver as a major site of cholesteryl ester hydrolysis in the rat, the *in vivo* role of microsomal CEH calls for further study. Regulation of neutral microsomal CEH by protein phosphorylation cannot be confirmed without examination at the cellular and molecular level. Purification of the enzyme and studies of phosphorylation in isolated rat liver cells are the subject of ongoing investigations.

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Biphasic Modulation of Choline Uptake and Phosphatidylcholine Biosynthesis by Vasopressin in Rat Cardiac Myocytes

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The effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis in isolated rat heart myocytes was investigated. Myocytes were incubated with labelled choline in the presence of 0.05–1.0 μM vasopressin. Uptake of choline was enhanced (25%) by a low concentration (0.2 μM) of vasopressin, but was attenuated (19%) by a higher vasopressin concentration (1.0 μM). The biosynthesis of phosphatidylcholine was also affected by vasopressin in a biphasic manner. At low concentrations of vasopressin, a general increase in cytosine triphosphate:phosphocholine cytidylyltranferase activity was observed that caused an enhanced conversion of phosphocholine to phosphatidylcholine *via* the cytidine diphosphocholine pathway. At high vasopressin concentrations, a decrease in the activity of cytidylyltransferase was detected, which was caused by the translocation of the enzyme from the microsomal fraction to the cytosolic fraction. The decrease in enzyme activity coincides with a reduction in the conversion of labelled phosphocholine to phosphatidylcholine. In view of the fact that phospholipid biosynthesis in rat hepatocytes is inhibited by vasopressin at all concentrations, the biphasic modulation of phosphatidylcholine biosynthesis in rat heart myocytes illustrates the diverse effects of this hormone in different mammalian tissues.
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Phosphatidylcholine is the principal phospholipid in mammalian tissues (1). In mammalian hearts, the vast majority of phosphatidylcholine is synthesized *via* the cytidine diphosphocholine (CDPcholine) pathway (2,3). In this pathway, choline taken up by the heart is rapidly phosphorylated into phosphocholine by the action of choline kinase. Phosphocholine is then converted into CDPcholine by the action of cytosine triphosphate (CTP):phosphocholine cytidylyltransferase. The CDPcholine is condensed with diacylglycerol by the action of CDPcholine:diacylglycerol cholinephosphotransferase to form phosphatidylcholine. Although the first committed step in this pathway is catalyzed by choline kinase, the rate-limiting step is catalyzed by the cytidylyltransferase (4). The latter enzyme is located in both microsomal and cytosolic fractions, and translocation of the enzyme from one compartment to another is regarded as an important mechanism for the *in vivo* regulation of enzyme activity (5).

The effects of hormones on phosphatidylcholine biosynthesis have been investigated in mammalian tissues. Steroids, including diethylstilbestrol and glucocorticoids, have been found to stimulate phosphatidylcholine biosynthesis in the rooster and fetal lung (6–8). Alternatively, norepinephrine and vasopressin have been shown to inhibit phosphatidylcholine biosynthesis (9–11). The inhibitory effect of vasopressin in hepatocytes is mediated *via* the modulation of the CTP:phosphocholine cytidylyltransferase activity without affecting the uptake or phosphorylation of choline (9). In another study, high levels of vasopressin appear to inhibit the CDPcholine:1,2-diacylglycerol cholinephosphotransferase activity through increased levels of intracellular calcium (10). Vasopressin has also been shown to exhibit biphasic effects in the perfused heart with respect to changes in peak ventricular pressure (12). However, the effect of vasopressin on phosphatidylcholine biosynthesis in cardiac cells has not been reported.

In this study, the effects of vasopressin on choline uptake and phosphatidylcholine biosynthesis in cardiac myocytes were investigated. Our results show that both choline uptake and phosphatidylcholine biosynthesis in myocytes were enhanced at low vasopressin concentrations, but that the biosynthesis of phosphatidylcholine is attenuated at high vasopressin concentrations.

MATERIALS AND METHODS

Materials. Phospho[*methyl*-^{14}C]choline, CDP[*methyl*-^{14}C]-choline, [*tyrosyl*-^{3}H]arginine and vasopressin were obtained from Amersham Canada Ltd. (Oakville, Ontario, Canada). The [*methyl*-^{3}H]choline was purchased from New England Nuclear Division of Dupont (Mississauga, Ontario, Canada). Oxytocin and [arginine]vasopressin were products of Boehringer Mannheim Canada Ltd. (Laval, Quebec, Canada). Minimal essential medium (Joklik-modified), Medium 199, Dulbecco's phosphate buffered saline, phosphocholine, choline, cytidine 5′-diphosphocholine, *tris*-base, CTP, adenosine triphosphate, hyaluronidase and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine (pig liver) and 1,2-diacylglycerol (pig liver) were purchased from Serdary Research Laboratory (London, Ontario, Canada). Magnesium acetate and magnesium chloride were products of Fisher Scientific Co. (Fair Lawn, NJ). Sephadex G-15 (fine) gel filtration media was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Collagenase (Class 2) was obtained from Worthington Biochemical Corporation (Freehold, NJ). All other chemicals were of analytical grade and obtained locally from the Canlab Division of Baxter Co. (Edmonton, Alberta, Canada) Sprague Dawley rats of either sex, 250 ± 50 g, were obtained from Charles River Canada, Inc. (St. Constante, Quebec, Canada).

Isolation of rat cardiac myocytes. Myocardial cells were obtained from hearts of Sprague Dawley rats. Rats were maintained on Agway rodent chow and tap water *ad libitum*. Myocytes were isolated by the procedure of Langer *et al.* (13). Briefly, the heart was perfused in

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Abbreviations: CDPcholine, cytidine diphosphocholine; CTP, cytosine triphosphate; EDTA, ethylenediaminetetraacetic acid; MEM, minimum essential medium.

Langendorff mode with buffer A for 5–10 min followed by perfusion with buffer B for another 15–20 min. Following perfusion, the digested tissue was placed in buffer A and the cells were dislodged from the tissue by gentle agitation. Myocytes were suspended with buffer A in a culture tube and separated by sedimentation and removal of supernatant containing other cardiac cells. The myocyte preparation, which was relatively free of other cardiac cells, was resuspended in a small volume of buffer A, and two volumes of the growth medium (containing 1.8 mM calcium) were slowly added to the myocyte suspension over a period of 45 min. Finally, myocytes were placed in the growth buffer, incubated in a petri dish for 60 min in order to allow attachment and used immediately after incubation. All incubations were carried out at 37°C and all the buffers used were saturated with 95% oxygen/5% carbon dioxide. Buffer A contained 11 g/L minimum essential medium-Jokliks/(MEM-Jokliks) buffer preparation, 60.0 mM taurine, 2.0 mM DL-carnitine, 1.0 mM adenosine, 3.4 mM magnesium chloride, 50.0 mM sodium bicarbonate, 15.0 mM glucose, 0.1 mM octanoic acid, 8.0 mM L-glutamate, 0.1 g/L penicillin and 0.1 g/L streptomycin. Buffer B contained 4 mg/mL collagenase (class 2), 2 mg/mL hyaluronidase and 0.1 mM calcium chloride dissolved in buffer A. The growth medium contained 9.8 g/L of Medium 199, 2.0 mM L-glutamine, 2.2 g/L sodium bicarbonate, 0.1 g/L penicillin and 0.1 g/L streptomycin. The viability of the myocytes was tested by trypan blue exclusion and contractile activity. Over 90% of the cells isolated were viable and in rod shape throughout the experiment.

The determination of choline uptake and analysis of choline-containing metabolites. Myocytes were used immediately after attachment to the petri dish. Each dish (containing 200–300 μg protein) was incubated with 2 mL of growth medium containing 10 μM labelled choline (0.15 mCi/μmol) for the prescribed time period. The medium containing labelled choline was removed, and the cells were washed twice with 2 mL of Dulbecco's phosphate buffered saline followed by the addition of 1 mL methanol. The cell suspension was transferred to a glass test tube, and an equal volume of chloroform was added. An aliquot of the mixture was taken for the determination of total choline uptake. Lipid extraction of the myocytes was performed by the method of Folch *et al.* (14). Subsequent to phase separation, the phosphatidylcholine fraction in the lower phase was isolated by thin-layer chromatography on silica G-25 plates with a solvent containing chloroform/methanol/water/acetic acid (70:30:4:2, by vol). Choline-containing metabolites in the upper phase were separated by thin-layer chromatography with a solvent system containing methanol/0.6% sodium chloride/57% ammonium hydroxide (50:50:5, by vol). The locations of the radioactive fractions on the thin-layer chromatographic plate were detected by a Bioscan System 200 Imaging Scanner.

The determination of enzyme activities. Choline kinase activity was assayed by determining the conversion of [*methyl*-^{3}H]choline to phosphocholine (15). CTP:phosphocholine cytidylyltransferase activity was assayed by the conversion of phospho[*methyl*-^{14}C]choline to CDPcholine (16). The reaction mixture contained 100 mM Tris-succinate (pH 7.5), 12 mM magnesium acetate, 2.5 mM CTP, 1.0 mM phospho[*methyl*-^{14}C]choline and enzyme preparation to a final volume of 100 μL. A total cardiac phospholipid extract (containing 0.1 μM lipid phosphorus) was added to the assay mixture when the cytosolic fraction was used. The reaction was carried out at 37° for 20 min and stopped by boiling. The labelled CDPcholine was separated from labelled substrate by thin-layer chromatography with a solvent system containing methanol/0.6% aqueous sodium chloride/57% ammonium hydroxide (50:50:5, by vol). CDPcholine:1,2-diacylglycerol cholinephosphotransferase activity was determined by the conversion of CDP[*methyl*-^{14}C]choline to phosphatidylcholine (17). The reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 1 mM EDTA, 0.4 mM CDP[*methyl*-^{14}C]choline, 1.0 mM diacylglycerol (prepared in 0.015% Tween 20 by sonication) and enzyme preparation to a final volume of 1.0 mL. The reaction was carried out at 37° for 20 min and terminated by the addition of 3 mL of chloroform/methanol (2:1, vol/vol) to the mixture. Water (0.5 mL) was added to cause phase separation. The lower phase (containing the labelled phosphatidylcholine) was washed twice with 40% methanol, and an aliquot was taken for radioactivity determination.

Subcellular fractionation. Microsomal and cytosolic fractions were obtained from cardiac myocytes by differential ultracentrifugation. Myocytes were suspended in 10 mM Tris-HCl (pH 7.4)/0.25 M sucrose/1 mM EDTA and homogenized in a glass Dounce homogenizer. Myocyte disruption after homogenization was verified under a phase contrast light microscope. The cellular suspension was centrifuged at 20,0000 × *g* for 10 min, and the supernatant obtained was centrifuged at 100,000 × *g* for 60 min. The supernatant (cytosol) was collected, and the precipitate (microsomal pellet) was resuspended in the homogenizing buffer.

Other analytical procedures. Protein was determined by the method of Lowry *et al.* (18) with bovine serum albumin as standard. Radioactivity was determined by liquid scintillation counting. Student's *t*-test was used for statistical analysis of data. The significance level was set at $P < 0.05$.

RESULTS

The effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis. Cardiac myocytes were incubated in the growth medium containing 10 μM [*methyl*-^{3}H]choline in the presence of 0.2 and 1.0 μM [arginine]vasopressin for 20–120 min. Myocytes incubated in the same medium without vasopressin were used as controls. Subsequent to incubation, total uptake of choline by myocytes was determined. As depicted in Figure 1, choline uptake by myocytes was linear up to 60 min in the presence or absence of vasopressin. The presence of 0.2 μM vasopressin caused an enhancement of choline uptake (25%) at all time points, whereas the presence of 1 μM vasopressin caused a significant reduction (19%) in choline uptake.

The effect of other vasopressin concentrations (0.01–5 μM) on choline uptake and phosphatidylcholine biosynthesis was investigated. Myocytes were incubated with growth medium containing 10 μM labelled choline for 60 min at various vasopressin concentrations. Subsequently, the uptake of choline and the amount of radioactivity in the phosphatidylcholine fraction in the myocytes were determined. No change in the uptake of choline was detected at 0.01 μM vasopressin. An enhancement of choline

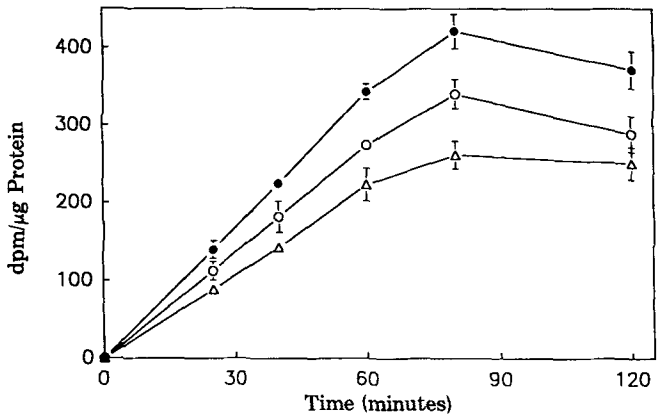

FIG. 1. Time course of choline uptake in rat cardiac myocytes. Rat cardiac myocytes were incubated with 10 μM [*methyl*-^{3}H]choline (150 μCi/μmol) in the absence (○) or presence of 0.2 μM (●) or 1.0 μM (△) vasopressin. The total amount of radioactivity incorporated into the myocytes at each time point was determined. Each point represents the mean value of four determinations from two separate experiments, and the vertical bar represents the SD.

uptake was observed with a corresponding increase in the labelling of phosphatidylcholine at 0.05–0.3 μM vasopressin (Table 1). At higher vasopressin concentrations (0.4–1.0 μM), choline uptake was attenuated, together with a reduction in the labelling of phosphatidylcholine. It appears that choline uptake in myocytes was modulated by vasopressin in a biphasic manner, with a transition occurring between 0.3–0.4 μM. However, the change in the labelling of phosphatidylcholine was not proportional to the change in choline uptake, which indicates that vasopressin might also affect phosphatidylcholine biosynthesis in myocytes.

The nature of vasopressin response was investigated by using a specific V_1 receptor antagonist [d(CH_2)$_5$, D-tyr(OEt)^2, val^4, cit^8]-vasopressin. Myocytes were incubated with 10 μM [*methyl*-^{3}H]choline with 0.2 μM vasopressin for 60 min in the presence or absence of 0.1 μM vasopressin antagonist. As shown in Table 1, both the choline uptake and the labelling of phosphatidylcholine were not affected by the vasopressin antagonist. However, the stimulatory effect of 0.2 μM vasopressin on choline uptake and labelling of phosphatidylcholine was attenuated by the presence of the vasopressin antagonist.

Analysis of choline-containing metabolites. The effect of vasopressin on the radioactivities associated with the choline-containing metabolites in myocytes was determined. The choline-containing metabolites in the upper phase of the cellular extract were separated by thin-layer chromatography with a solvent system containing methanol/0.6% sodium chloride/57% ammonium hydroxide (50:50:5, by vol). In the presence of 0.2 μM vasopressin, the increase in the labelling of choline (24%) commensurated with the increase in choline uptake (Table 2). However, the increase in the labelling of phosphocholine (15%) was less than that shown in choline uptake. In the presence of 1 μM vasopressin, the decrease in choline uptake was also reflected in the labelling of choline (23%), but not in the labelling of phosphocholine. The labelling of CDPcholine was not significantly changed in both cases.

TABLE 1

Effect of Vasopressin on Choline Uptake and Incorporation of Labelled Choline into Phosphatidylcholine[a]

Hormone	Total choline uptake (dpm/μg protein)	Phosphatidylcholine (dpm/μg protein)
Control	274 ± 8 (5)	68 ± 3 (8)
0.05 μM vasopressin	301 ± 10 (5)[b]	79 ± 3 (8)[b]
0.1 μM vasopressin	304 ± 14 (3)[b]	82 ± 3 (3)[b]
0.2 μM vasopressin	343 ± 10 (5)[b]	97 ± 11 (6)[b]
0.3 μM vasopressin	324 ± 15 (3)[b]	73 ± 9 (3)
0.4 μM vasopressin	229 ± 7 (3)[b]	58 ± 8 (3)[b]
0.5 μM vasopressin	323 ± 19 (3)[b]	60 ± 2 (3)[b]
1.0 μM vasopressin	223 ± 21 (4)[b]	41 ± 4 (9)[b]
0.1 μM V_1 antagonist	267 ± 8 (8)	68 ± 4 (3)
0.1 μM V_1 antagonist +0.2 μM vasopressin	287 ± 3 (3)	74 ± 6 (4)

[a]Cardiac myocytes were incubated with growth medium containing 10 μM [*methyl*-^{3}H]choline (150 μCi/μmol) for 60 min in the presence of vasopressin and 0.1 μM [d(CH_2)$_5$, D-tyr(OEt)^2, val^4, cit^8] vasopressin (V_1 antagonist). Subsequently, the total choline uptake and the incorporation of label into phosphatidylcholine were determined. Each set of values represents the mean ± SD (number of experiments).
[b]$P < 0.05$.

TABLE 2

Effect of Vasopressin on the Incorporation of Radioactivity into Choline-Containing Metabolites[a]

Vasopressin	Choline (dpm/μg protein)	Phosphocholine (dpm/μg protein)	CDPcholine (dpm/μg protein)
Control	53 ± 3 (6)	163 ± 9 (4)	8.8 ± 2.9 (6)
0.2 μM	66 ± 4 (6)[b]	187 ± 14 (5)[b]	9.4 ± 1.8 (3)
1.0 μM	41 ± 11 (13)[b]	152 ± 7 (3)	6.8 ± 2.6 (9)

[a]Cardiac myocytes were incubated with growth medium containing 10 μM [*methyl*-^{3}H]choline for 60 min in the presence of 0.2 or 1.0 μM vasopressin. Subsequently, the radioactivity associated with the choline-containing metabolites of the cytidine diphosphocholine (CDPcholine) pathway in the myocytes were determined. Each set of values represents the mean ± SD (number of experiments).
[b]$P < 0.05$.

Activities of enzymes in the CDPcholine pathway. The disproportionate changes between choline uptake and labelling of phosphatidylcholine suggest that the conversion of metabolites in the CDPcholine pathway was affected by vasopressin. This postulation was substantiated by the analysis of the choline-containing metabolites. In order to elucidate the mechanism leading to these changes, the activities of the enzymes in the CDPcholine pathway were determined. Myocytes were homogenized to yield a cellular homogenate for enzyme activity determinations. The presence of 0.2 or 1.0 μM vasopressin to the assay mixture had no effect on any of the enzyme activities. In another set of experiments, myocytes were incubated with 0.2 or 1.0 μM vasopressin for 60 min prior to homogenization. Myocytes incubated in the absence of vasopressin were used as controls. The activities of choline kinase, CTP:phosphocholine cytidylyltransferase and CDPcholine:diacylglycerol cholinephosphotransferase were determined and results obtained are shown in Table 3. Incubation with 0.2 μM vasopressin caused a twofold enhancement of the cytidylyltransferase activity, but the

TABLE 3

The Effect of Vasopressin on the Activities of the Phosphatidylcholine Biosynthetic Enzymes[a]

Vasopressin	Choline kinase (nmol/min/mg protein)	CTP:phosphocholine cytidylyltransferase (nmol/min/mg protein)	Cholinephosphotransferase (nmol/min/mg protein)
Control	0.018 ± 0.003 (4)	0.254 ± 0.035 (8)	0.116 ± 0.012 (8)
0.2 μM	0.018 ± 0.005 (3)	0.495 ± 0.065 (5)[b]	0.119 ± 0.008 (5)
1.0 μM	0.015 ± 0.002 (3)	0.118 ± 0.017 (4)[b]	0.124 ± 0.007 (5)

[a]Cardiac myocytes were incubated with growth medium in the presence or absence of vasopressin. Subsequently, the myocytes were homogenized, and enzyme activities in the homogenate were determined. Each set of values represents the mean ± SD (number of experiments). CTP, cytosine triphosphate.
[b]$P < 0.05$.

activities of other enzymes in the pathway were not affected. Incubation with 1.0 μM vasopressin caused a severe (54%) reduction in the cytidylyltransferase activity. The activities of choline kinase and cholinephosphotransferase were not significantly changed by this treatment.

The mechanism for the change of cytidylyltransferase activity at different vasopressin concentrations was examined. Myocytes incubated with 0.2 or 1.0 μM vasopressin were homogenized, and subcellular factions were obtained by differential centrifugation. Enzyme activities in the microsomal and cytosolic fractions were determined, and the results are depicted in Table 4. In the presence of 0.2 μM vasopressin, enhancement of enzyme activities in both the microsomal and cytosolic fractions was observed. In the presence of 1.0 μM vasopressin, there was a severe reduction in enzyme activity in the microsomal fraction, with a corresponding increase in enzyme activity in the cytosolic fraction.

The uptake of vasopressin in cardiac myocytes. The ability of the cardiac myocytes for vasopressin uptake was investigated. Myocytes were incubated with 0.2 or 1.0 μM [^{3}H-tyr]vasopressin (1.5 μCi/nmol) for 60 min in growth medium followed by two washings with growth buffer containing 10 μM nonlabelled vasopressin. After incubation, the cells were homogenized in 0.25 M sucrose/10 mM Tris-HCl buffer (pH 7.5), and an aliquot of the homogenate was taken for radioactivity determination (Table 5). The uptake of vasopressin by the myocytes was found to be dose-dependent, but not in a linear fashion. The fate of the vasopressin taken up by the cells was also examined.

TABLE 4

Effect of Vasopressin on the Distribution of CTP:Phosphocholine Cytidylyltransferase in the Microsomal and Cytosolic Fractions[a]

Vasopressin	Cytidylyltransferase activity	
	Microsomal (nmol/min/mg protein)	Cytosolic (nmol/min/mg protein)
Control	0.415 ± 0.073 (4)	0.319 ± 0.086 (3)
0.2 μM	0.612 ± 0.071 (3)[b]	0.543 ± 0.048 (3)[b]
1.0 μM	0.169 ± 0.029 (3)[b]	0.605 ± 0.041 (3)[b]

[a]Cardiac myocytes were incubated with growth medium in the presence or absence of vasopressin. Subsequently, the myocytes were homogenized, and subcellular fractions were obtained by differential centrifugation. The cytosolic enzyme activity was assayed in the presence of phospholipids. Each set of values represents the mean ± SD (number of experiments). CTP, cytosine triphosphate.
dose-dependent, but not in a linear fashion. The fate of the
[b]$P < 0.05$.

TABLE 5

The Uptake of Vasopressin by Rat Cardiac Myocytes[a]

Vasopressin in medium	Vasopressin uptake (fmol/μg protein)
0.2 μM	5.5 ± 1.1 (6)
1.0 μM	22.2 ± 8.0 (6)

[a]Cardiac myocytes were incubated in the presence of [^{3}H-tyr]vasopressin (1.5 μCi/nmol) for 60 min. After incubation, medium was removed and cells were washed with unlabelled vasopressin (10 μM). The myocytes were homogenized and the amount of radioactivity in the homogenate was determined. Each set of values represents the mean ± SD (number of experiments).

The myocyte homogenate was centrifuged at 100,000 × *g* for 60 min to obtain the cytosolic fraction, and an aliquot was applied to a Sephadex G-15 (fine) column equilibrated with 10 mM Tris-HCl (pH 7.5)/0.1 M KCl. In a control experiment, vasopressin was applied and eluted from the same column. None of the radioactivity was found in the fractions that contained vasopressin, but most of the radioactivity was eluted in subsequent fractions that normally contained tyrosine (data not shown). It appears that the vasopressin taken up by the cell was metabolized into the corresponding labelled amino acid and its metabolites.

DISCUSSION

It is clear from this study that vasopressin affects phosphatidylcholine biosynthesis in isolated myocytes. Two modes of regulation have been identified: (i) the modulation of choline uptake and (ii) the regulation of the CTP:phosphocholine cytidylyltransferase activity.

The uptake of choline by the cell has been regarded as a plausible mechanism for the control of phosphatidylcholine biosynthesis. In nonneural cells, choline is taken up *via* the low-affinity Na^+-independent transport system (19). In the mammalian heart, the uptake of choline follows Michaelis-Menten kinetics and appears to consist of a single uptake system (2,20). The uptake of choline is inhibited by ethanolamine and other choline analogs (21,22). Interestingly, choline uptake is enhanced by amino acids in some mammalian tissues (20,23). This is the first report that choline uptake could also be regulated by hormonal control. The modulation of choline uptake by vasopressin appears to be quite specific as the action of vasopressin could not be duplicated by similar

amounts of oxytocin (data not shown). Experiments conducted with [d(CH_2)$_5$, D-tyr(OEt)^2, val^4, cit^8]-vasopressin, a specific V_1 receptor antagonist (24), indicate that the action of vasopressin might be associated with the V_1 receptors.

The disproportionate changes between choline uptake and radioactivity incorporated into the phosphatidylcholine fraction suggest that the biosynthesis of the phospholipid is also modulated by vasopressin. It is interesting to note that vasopressin at lower concentrations (0.05–0.3 μM) regulates choline uptake and the synthesis of phosphatidylcholine in a synergistic manner. Analyses of the choline-containing metabolites revealed that the conversion of phosphocholine to CDPcholine and, subsequently, phosphatidylcholine, was enhanced, and the cytidylyltransferase activities were elevated in both the microsomal and cytosolic fractions. In order to determine whether the enzyme might be modulated directly by the hormone, the ability of the myocytes to take up vasopressin was examined. Only a very small proportion (fmol/μg cellular protein) of vasopressin was internalized by the myocytes, and practically all of the internalized vasopressin was catabolized into amino acids and their metabolites. Furthermore, the addition of vasopressin to the assay mixture *in vitro* did not cause any changes in the cytidylyltransferase activity. Hence, the modulation of the cytidylyltransferase was not a direct interaction between the enzyme and vasopressin.

One intriguing aspect of this study is the biphasic action of vasopressin on both choline uptake and phosphatidylcholine biosynthesis. In view of the fact that the concentrations of vasopressin used in this study are much higher than the physiological values, the role of the hormone in the regulation of phosphatidylcholine biosynthesis *in vivo* remains undefined. The V_1 receptor is the predominant type of vasopressin receptors in nonrenal organs and the ability of vasopressin to activate protein kinase C at high concentrations *via* the V_1 receptor is well documented (24). However, it is not clear if the protein kinase C produced by the action of vasopressin may cause the phosphorylation of the cytidylyltransferase, which results in its translocation from the microsomal to the cytosolic form (5,25). Alternatively, vasopressin at lower concentrations may produce a yet unidentified cellular response that activates the cytidylyltransferase in both subcellular fractions. The ability of a hormone to produce dual second messengers *via* a single receptor has recently been identified (26,27). Interestingly, vasopressin displays no effect on the choline uptake in isolated rat hepatocytes, but inhibits the incorporation of labelled choline into phosphatidylcholine (9). Similar to the present study, the mechanism of inhibition appears to be at the cytidylyltransferase level. Hence, the biphasic effect of vasopressin on choline uptake and phosphatidylcholine biosynthesis observed in this study illustrates the diverse effects of this hormone on different tissues.

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Isolation, Characterization and Biological Activity of Inositol Sphingophospholipids from *Phytophthora capsici*[1]

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Several inositol sphingophospholipids (ISPL) were isolated from mycelia of *Phytophthora capsici*, a phytopathogenic fungus of pepper (*Capsicum annuum* c.v. Yolo Wonder). The sphingophospholipids induce a protective response by cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici*. The ISPL structures were determined by chemical methods, fast atom bombardment mass spectrometry and by fast atom bombardment collision-induced tandem mass spectrometry. All ISPL consisted of ceramides linked to inositol phosphate. The amide-linked fatty acids were found to have 16, 20 and 22 carbon chains. The long chain bases were identified as trimethylsilyl derivatives and shown to be C_{16}-sphingosine and C_{16}-dihydrosphingosine. The protective effect of the ISPL on cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici* was shown not to be dependent on the unsaturation of the C_{16}-sphingosine base and the nature of the acyl chain. We also investigated the effect of growth conditions on the ISPL produced in three different, chemically defined media that are commonly used for fungi cultures. We showed that *P. capsici* synthesized one major class of compounds with molecular weights of M_r = 833 and 835 containing the 16:1 base and the 22:1 or the 22:0 *N*-acyl group, independent of the growth medium used. The presence of phosphite or fosetyl-Al did not affect the result.

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Various lipids from parasitic fungi have been shown to be elicitors of plant defense reactions (1–3). We have previously reported that lipid components of *Phytophthora capsici* mycelia induced protection in pepper (*Capsicum annuum* c.v. Yolo Wonder) against the pathogen (4). The most active lipid fraction was identified as inositol sphingophospholipids (ISPL) (5). ISPL have not previously been shown to occur in *P. genus*.

Earlier studies had shown that ISPL are cell membrane components which appear to play an important role in host-parasite interactions (6). ISPL have been found in plants (7–11), yeasts (12–14), fungi (15), and protozoa (6,16,17), and all were shown to be derived from myoinositol phosphorylceramide (6,13). The ceramides typically contain C_{16} or C_{18} sphingosine or C_{18} phytosphingosine (5,6,13) as long chain base (LCB), which is acylated with palmitic, stearic (6,16), 2- or 3-hydroxyhexacosanoic or 4-hydroxy-2-docosenoic acid (5,13) to form the backbone of the glycosphingophospholipids (18).

[1]This work is dedicated to Dr. P.M. Molot.

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Abbreviations: CID, collision-induced dissociation; FAB, fast atom bombardment; FAME, fatty acid methyl esters; GC, gas chromatography; HPTLC, high-performance thin-layer chromatography; ISPL, inositol sphingophospholipid; LCB, long chain base; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

Lhomme *et al.* (5) have shown that *P. capsici* strains 15-12A and 197 synthesize nonglycosylated ISPL. Because these ISPL are biologically active by rendering plants resistant to pathogenic fungi, it was of interest to determine whether inositol sphingophospholipids were present in other strains of *P. capsici* and whether their composition was dependent on growth conditions.

It had previously been shown that phosphite, the active breakdown product of fosetyl-Al in plant tissues (19), stimulates plant defense mechanisms (20,21). Thus, it was important to determine whether sphingophospholipid structure is affected when the fungus is grown in either phosphite or fosetyl-Al containing media.

MATERIALS AND METHODS

Microorganisms and growth. Three strains, 107, 197 and 375, of *P. capsici* were grown on various media; strain 107 on M1, M2 and M3 media; strain 197 on M1 and M2 media; and strain 375 on M1, M4 and M5 media.

M1 was a chemically defined medium (22) which contained (g/L): sucrose, 50; malt extract (crystallized), 5; $Ca(NO_3)_2$, 2; KNO_3, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.5; $FeSO_4$, $MnSO_4 \cdot H_2O$, Cu $SO_4 \cdot 5H_2O$ and Zn $SO_4 \cdot 7H_2O$, 0.001; citric acid, 0.025; malic acid, 0.025; malic acid, 0.025; boric acid, 0.015; with pH adjusted to 4.5. M2 medium had the same composition as M1 but was supplemented with 10 mg/L fosetyl-Al. M3 was the Plich and Rudnicki medium (23) which contained (g/L): glucose, 25; yeast extract, 5; L-asparagine, 1; thiamine, 0.001; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1; with pH adjusted to 6. M4 was the Ribeiro synthetic medium modified according to Fenn and Coffey (24), which contained (g/L): glucose, 10; L-asparagine, 0.1; KNO_3, 0.15; KH_2PO_4, 1; $CaCl_2$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.004; $ZnSO_4 \cdot 7H_2O$, 0.009; $CuSO_4 \cdot 5H_2O$, 0.008; $MnSO_4 \cdot H_2O$, 0.001; $Na_2B_4O_7$, 0.0005; $FeCl_3 \cdot 6H_2O$, 0.005; ethylenediaminetetraacetic acid, 0.026; KOH, 0.015; thiamine, 0.001; with pH adjusted to 6.5. M5 medium was M4 medium supplemented with 5 mM disodium phosphite and 0.8 mM KH_2PO_4.

Fungi were grown at 24°C under 16 h of light on M1, M2, M4 and M5 media. Strain 107 was grown in the dark on M3 medium. Mycelia were collected after three weeks of growth.

Extraction and fractionation of lipids. Mycelial walls were isolated from fungi as described by Fabre *et al.* (25), and were treated with chloroform/methanol (2:1, vol/vol). The extracted lipids were separated by column chromatography on Bio-Sil HA silicic acid (BioRad, Ivry sur Seine, France) with chloroform and chloroform containing increasing concentrations of methanol as eluents. ISPL were eluted with chloroform/methanol (50:50, vol/vol) and purified further by column chromatography on DEAE-cellulose according to Rouser *et al.* (26). ISPL were eluted with a mixture containing 50 parts of a 0.04 M ammonium acetate solution in chloroform/methanol (4:1, vol/vol) and 1 part of 29% ammonia solution.

Thin-layer chromatography of lipid fractions eluted from the silicic acid column. Lipid fractions were analyzed by high-performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck, Darmstadt, Germany) that were developed with chloroform/methyl acetate/*n*-propanol/methanol/0.25% aqueous KCl (25:25:28:10:7, by vol) (27). Fractions were made visible by spraying with 0.2% vanillin (wt/vol) in 10% sulfuric acid (vol/vol).

Phospholipids were analyzed by two-dimensional HPTLC with the solvent system described by Rouser *et al.* (28). Fractions were detected by spraying with a Dittmer and Lester reagent as modified by Vaskovsky and Kostetsky (29) and identified by comparison with authentic standards.

Analytical methods. Phospholipids were hydrolyzed with 4 N or 6 N HCl for 48 h at 100°C. Fatty acids were recovered from the hydrolysate by chloroform extraction and esterified with diazomethane. Inositol was acetylated with acetic anhydride/pyridine (1:1, vol/vol) at 100°C for 15 min before analysis by gas chromatography.

Sphingosines were released by methanolysis according to Laine *et al.* (30), and their *N*-acetyl derivatives were prepared by addition of a mixture of methanol/acetic anhydride (4:1, vol/vol) and reaction overnight at room temperature. Silylation of hydroxyl groups was performed with the *N,O-bis*(trimethylsilyl)trifluoracetamide silylating reagent for 30 min at 60°C. Phosphorus was measured by the colorimetric method of Lowry *et al.* (31).

Gas chromatography. Fatty acid methyl esters (FAME) were separated by gas chromatography (GC) using an Intersmat instrument (Model 120FL; Intersmat, Lyon, France) fitted with an SP2100 glass capillary column (60 m × 0.32 mm). The column temperature was programmed from 140 to 260°C at 4°C/min. An SP2380 glass capillary column (30 m × 0.32 mm) was used for analysis of hexaacetylinositol.

Combined GC/mass spectrometry (GC/MS) of trimethylsilyl-*N*-acetyl-sphingosines was performed on a Ribermag R10 (Nermag SA, Rueil-Malmaison, France) apparatus which was connected to a gas chromatograph (Hewlett-Packard 437; Hewlett-Packard, Les Ulis, France) equipped with an OV-1 capillary column (25 m × 0.32 mm). The column temperature was programmed from 210 to 250°C at 2°C/min.

GC/MS of FAME was performed on a UG-Micromass 305 apparatus equipped with a DB-1 capillary column (30 m × 0.3 mm). Oven temperature was held at 60°C for 1 min, then increased at 30°C/min to 120°C, and then at 2°C/min to 180°C where it was maintained for 2 min.

MS. Fast atom bombardment (FAB) mass spectra of phospholipids were recorded in the negative ion mode on a VG ZAB2 SEQ mass spectrometer (Manchester, United Kingdom). The instrument was equipped with a VG FAB standard source with a cesium ion gun delivering about 2 μA of cesium ion current at about 35 kV.

Spectra were obtained at a magnet scan rate of 20 s/decade over a mass range of *m/z* 200–2000 at 8 kV. The mass range was calibrated using cesium iodine clusters. The spectra were collected and processed using the VG II-250 J data system. Samples were dissolved in chloroform/methanol (1:1, vol/vol) and mixed with an equal volume of triethanolamine. The sample (2 μL) was deposited on the FAB probe tip mixed with matrix, *i.e.*, glycerol.

Low-resolution FAB and FAB collision-induced decomposition (CID) tandem mass spectra (MS/MS) were obtained with a JEOL HX110/HX110 tandem mass spectrometer (Tokyo, Japan), operated at ±10 kV accelerating voltage; with ±18.5 kV post-acceleration at the detector. The JEOL DA-5000 data system was used for instrument control and data acquisition and processing.

Elicitor activity assay. Cotyledons from young peppers (*C. annuum* c.v., Yolo Wonder, susceptible to *P. capsici*) were removed and placed in water. Onto the lower side of the cotyledon, 25 μL of the elicitor fraction were added. On the same site, after 24 h, the cotyledons were infected using a 10-μL zoospore suspension (62550 zoospores/mL) that was produced as previously described (32). After four days of incubation at 22°C at a 16-h light cycle, the symptoms of infection were noted by the necrosis of whole cotyledons. Each trial involved 15–20 cotyledons. The cumulated results were expressed as susceptibility percentage corresponding to percentage necrotic cotyledons.

RESULTS

Lipids of P. capsici. The percentages of total lipids extracted from the mycelial cell walls are given in Table 1. The differences observed indicate that for strains 107 and 197, the lipid content is somewhat affected by the nature of the growth medium. *P. capsici*, strain 375, is characterized by a large total lipid content.

Total lipids were fractionated on a column of Bio-Sil HA silicic acid to measure the relative amounts of neutral and polar lipids (Table 1). The polar lipids consisted of phospholipids as revealed by HPTLC and by spraying with phosphorus reagent. Two-dimensional HPTLC showed the presence of a major compound that comigrated with ISPL, as previously described (5). The polar lipid fraction was then further purified by DEAE cellulose chromatography.

ISPL content. ISPL was identified by acid hydrolysis and methanolysis according to Laine *et al.* (30), followed by the identification of inositol, FAME and a compound that comigrated with standard sphingosine (R_f 1.14) in HPTLC using the solvent system described by Heape *et al.* (27). The inositol moiety was identified by GC as hexaacetyl derivative, and confirmed by FAB MS analysis of intact ISPL. The sphingosine moiety was characterized by GC/MS of the *N*-acetyl-*O*-trimethylsilyl derivative. The mass spectrum was identical to that of a C_{16} sphingosine and C_{16} dihydrosphingosine mixture described in 1966 (33) with molecular ions at *m/z* 283 and *m/z* 285, a peak at *m/z* 174 from the cleavage between carbon atoms 2 and 3, and a small peak at *m/z* 103 due to the terminal CH_2-O-Si$(CH_3)_3$ ion.

The FAMEs of the polar lipid fraction when analyzed by GC/MS (Table 2) were identified as hexadecanoic (16:0), octadecenoic (18:1), octadecanoic (18:0), eicosanoic (20:0), docosenoic (22:1), docosanoic (22:0) and hydroxydocosenoic (22h:1) acids. Fatty acid analyses of the polar lipid fraction from the various strains showed that their acyl moieties consisted of 16 to 22 carbon fatty acids, as previously reported (5,34). Although the same fatty acids are present in the three strains, there are significant differences in their relative abundance depending on the strain and the growth medium. This is especially true in regard to the C_{18}, C_{20} and C_{22} fatty acids. Thus, the

TABLE 1

Lipid Accumulation in Fungi[a]

P. capsici strain	Culture medium	Total lipid content[b] (%, w/w)	Percentage of each lipid fraction[c] (%, w/w)	
			Neutral lipids	Polar lipids
107	M1	9.5	76.5	23.5
	M2	9.2	68.0	32.0
	M3	11.3	69.9	30.1
197	M1	6.3	70.4	29.6
	M2	8.5	72.5	27.5
375	M1	29.6	68.4	31.6
	M4	10.1	67.6	32.4
	M5	10.7	75.8	24.2

[a]Three strains of *Phytophthora capsici* were examined. Strain 107 was grown on M1, M2 and M3 media. M1 was a chemically defined medium (Ref. 22); M2 has the same composition as M1 and was supplemented with 10 mg/L of foselyl-Al; M3 was the Plich and Rudnicki medium (Ref. 23). Strain 197 was grown on M1 and M2 media. Strain 375 was grown on M1, M4 and M5 media. M4 was the Ribeiro medium modified according to Fenn and Coffey (Ref. 24). M5 was the same medium supplemented with 5 mM disodium phosphite and with 0.8 mM KH_2PO_4. The compositions of M1, M3 and M4 media and the conditions of the cultures are described in the Materials and Methods section.
[b]These cultures were used to prepare the lipid extracts from fungal cell walls. The wt% of total lipid content is expressed as a percentage of the dry weight of fungal cell walls.
[c]The weight percentage of neutral and polar fractions from Bio-Sil HA silicic acid chromatography is expressed as a percentage of total lipids extracted.

TABLE 2

Fatty Acids from the Polar Lipid Fraction from *Phytophthora capsici*[a]

Fatty acid[b]	Abundance %[c]							
	Strain 107			Strain 197		Strain 375		
	M1	M2	M3	M1	M2	M1	M4	M5
Saturated								
Hexadecanoic (16:0)	46	44	33	46	37	51	41	55
Octadecanoic (18:0)	11	4	3	10	10	10	15	8
Eicosanoic (20:0)	6	8	7	10	14	5	10	11
Docosanoic (22:0)	4	13	21	15	10	5	25	13
Monounsaturated								
Octadecenoic (18:1)	20	4	2	4	5	6	0	1
Docosenoic (22:1)	12	19	32	13	20	21	10	7
Hydroxy and monounsaturated acid								
Hydroxydocosenoic (22h:1)	0	8	2	3	4	2	0	6

[a]Three strains of *P. capsici* were examined. See Table 1 for the composition of the growth media. The cultures were used to prepare the polar lipid fraction, which was hydrolyzed and used for the isolation of fatty acids.
[b]Fatty acids were analyzed by gas chromatography/mass spectrometry. For details see the Materials and Methods section.
[c]Data are given as percentage of total fatty acids present.

amounts of octadecanoic (18:0) and octadecenoic (18:1) acids in the strain 107 were higher in M1 medium than in M2 and M3 media, while the amounts of docosanoic (22:0) and docosenoic (22:1) acids were higher in a Plich medium (M3).

An increase in 22:0 fatty acid was also observed when strain 375 was grown on Ribiero medium (M4). Phosphite or fosetyl-Al (medium M5) only affected the percentage of saturated and unsaturated C_{22} fatty acids. In addition, hydroxydocosenoic acid (22h:1) appeared only in *P. capsici* grown in M5 medium.

Molecular species of ISPL. The negative ion FAB mass spectrum of the ISPL fraction from each strain showed two major molecular species with $[M - H]^-$ ions at *m/z* 832 and 834. As an example, Figure 1 shows the mass spectrum of the ISPL fraction from *P. capsici*, strain 197 (medium M1). The presence of other molecular species was indicated by ions at *m/z* 750, 752, 806, 808, 848 and 850.

In each spectrum, the ion $[Y_0PO_3H]^-$ corresponding to the cleavage of the inositol-phosphate bond, $[M - H - 162]^-$, and ions at *m/z* 241 and 259 were observed. Formation of these ions is shown in Figure 2 as previously reported (16,35,36). The fragments, assigned to inositol phosphate (37), provide evidence that the inositol and the ceramide are linked through a phosphate group.

In the CID MS/MS spectrum, additional fragments from the $[M - H]^-$ precursor ion were evident and

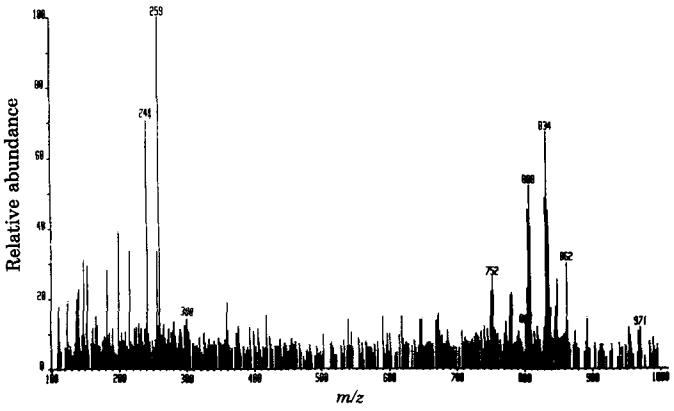

FIG. 1. The negative fast atom bombardment mass spectrum of inositol sphingophospholipids from *Phytophthora capsici* strain 197, grown on synthetic medium M1. The ions at *m/z* 752, 808 and 834 correspond to the individual molecular species present. Acyl groups and long chain bases were assigned to a particular molecular species in a mixed sample as confirmed by tandem mass spectrometry analysis of individual $[M - H]^-$ ions (see Table 3). Other fragments in the spectrum at *m/z* 241, 259 and 300 are characteristic of an inositol sphingophospholipid (see Fig. 2).

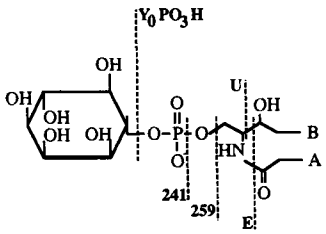

FIG. 2. Structures of major molecular species of inositol sphingophospholipids of molecular weights 833 (B = -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_n$-CH=CH-$(CH_2)_m$-CH_3, n + m = 18; E = 300; U = 620; Y_0PO_3H = 670) and 835 (B = -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{20}$-CH_3; E = 300; U = 622; Y_0PO_3H = 672) present in *Phytophthora capsici*. Fragmentations are characteristic of an inositol sphingophospholipid as reported by Dommon and Costello (Ref. 35).

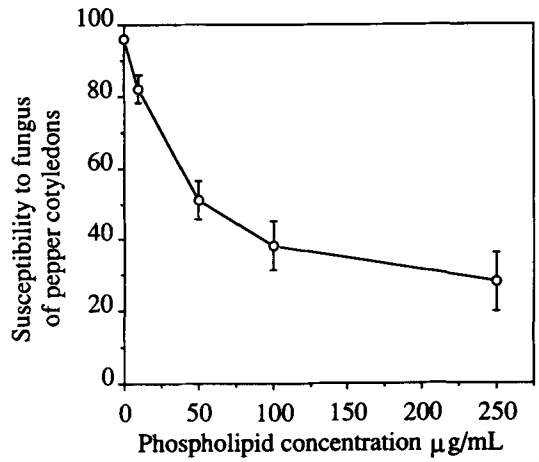

FIG. 3. Biological activity of inositol sphingophospholipids from *Phytophthera capsici* strain 197 grown on synthetic medium M1.

characterized by CID spectra of the ceramides, namely the ions E and the ions U (Fig. 2), as reported previously by Dommon and Costello (35). These ions were consistent with C_{16} sphingosine and C_{16} dihydrosphingosine containing ceramides. Analysis of the spectra made it possible to determine the LCB and the fatty acyl composition of the ceramide. The molecular species of ISPL in *P. capsici*, which differ in the unsaturation of the LCB and in the nature of the *N*-acyl chain, are summarized in Table 3. The result is in agreement with the structure (M_r = 833, M_r = 835) shown in Figure 2 for the most abundant ISPL of *P. capsici* strains 107, 197 and 375.

Biological activity. ISPL induce a protective mechanism by *C. annuum* (Yolo Wonder) against *P. capsici* (Fig. 3). When ISPL was applied onto *C. annuum* cotyledons at increasing concentrations, *i.e.*, 50–100 μg/mL of ISPL, the sensitivity of *Capsicum* cotyledons to the pathogen decreased by 70%. The same protective effect was observed when ISPL from each of the strains were examined. As an example (Fig. 3), an antinecrotic factor was detected in the ISPL fraction isolated from strain 197 grown on medium M2.

DISCUSSION

The present study confirms earlier observations that the major mycelial phospholipids in *P. capsici* are sphingophospholipids containing inositol (5). The structure

TABLE 3

Major Ions[a] in the CID Mass Spectra of Various Molecular Species of Inositol Sphingophospholipids from *Phytophthora capsici*

Molecular species $[M - H]^-$	IPO_4H_3 *m/z*	IPO_3H *m/z*	Y_0PO_3H *m/z*	U *m/z*	E *m/z*	LCB[b] identified	Fatty acid identified[c]
850	259	241	688	636	300	16:0	22h:1
848	259	241	686	636	300	16:1	22h:1
834	259	241	672	622	300	16:1	22:0
832	259	241	670	620	300	16:1	22:1
808	259	241	646	594	300	16:0	20:0
806	259	241	644	594	300	16:1	20:0
752	259	241	590	538	300	16:0	16:0
750	259	241	588	538	300	16:1	16:0

[a]The assignments of major ions of collisionally activated daughter spectra of the deprotonated molecular ions $[M - H]^-$ of inositol sphingophospholipids from *P. capsici* are based on interpretation of the collision-induced dissociation (CID) mass spectra of such compounds as reported by Domon and Costello (Ref. 35). An example of the fragmentations is given in Figure 2 for both major molecular species with $[M - H]^-$ ions at *m/z* 832 and 834 corresponding to the molecular weights 833 and 835.
[b]LCB, long chain base analogous to sphingosine and dihydrosphingosine identified based on ion $[M - H]^-$ and U ion (see Fig. 2).
[c]Identifications are based on both U and E ions (see Fig. 2).

of these inositol sphingophospholipids from *P. capsici* strains 107, 197 and 375 cultivated on different growth media were established by MS. FAB-CID-MS/MS was found to be a valuable technique for characterizing mixed molecular species composition of ISPL. The major ISPL contains a C_{16} dihydrosphingosine LCB linked *via* a docosanoyl (22:0) or docosenoyl (22:1) amide bond. The inositol and ceramide in turn, are linked through a phosphate group. Some differences among the strains of *P. capsici* were observed in the degree of unsaturation of the LCB and in the nature of the acyl group. The results confirm that ISPL produced by *P. capsici* are not glycosylated. They are similar to ISPL isolated from *Saccharomyces cerevisiae* (13) and *Leishmania dovani* (6).

With *P. capsici*, we observed only slight variations in the structure of ISPL with changes in the culture conditions. The only significant variation was in regard to the level of fatty acids with 22 carbon atoms.

Previously Lhomme *et al.* (5) have shown that in strains 15-12 and 197 of *P. capsici* grown on dextrose medium, the major ISPL corresponded to the compound of molecular mass 849, *i.e.*, an ISPL with 22h:1 fatty acid (Table 3), which was only a minor compound in the present systems. This difference suggests that the dextrose medium is not completely defined because strains 15-12 and 197 grown on a synthetic medium (M1) produced an ISPL with $M_r = 835$ as the major compound.

The biological activity of the sphingophospholipids had already been previously demonstrated (5). Our results indicate that the protective effect against the necrosis of young pepper cotyledons induced by the pathogen *P. capsici* is not dependent on the unsaturation of C_{16} sphingosine and on the nature of *N*-acyl side chain.

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Interaction of Dietary Fatty Acids and Cyclosporine A in the Borderline Hypertensive Rat: Tissue Fatty Acids

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In this study we examined (i) the effects of cyclosporine A (CS) on tissue lipid composition and (ii) the effect of changes in dietary n-6 fatty acids on tissue responses to CS. Fatty acid composition of liver, kidney, heart and brain were determined after 4 wk of treatment with CS (10 mg/kg•d p.o.) in male borderline hypertensive rats (BHR, n = 4/group), whose diet was supplemented with either safflower oil or evening primrose oil (EPO). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine/phosphatidylinositol, triglyceride and cholesteryl ester fatty acids were measured in kidney, heart, brain and liver. The same parameters were also measured in safflower-fed BHR (n = 4) receiving placebo. The effects of CS on liver microsomal Δ9, Δ6 and Δ5 desaturases *in vitro* were also followed. CS affected the fatty acid composition of all tissues examined, with the greatest changes seen in the renal phosphatidylcholine and phosphatidylserine/phosphatidylinositol fractions. All CS-induced changes that occurred in the liver, brain and renal fatty acids were reversed by EPO. CS elevated Δ9 desaturase but had no effect on Δ6 and Δ5 desaturase. In light of (i) the observation that EPO normalizes renal function and blood pressure in CS-treated BHR, and (ii) the importance of the kidney in blood pressure regulation, the data suggest that the beneficial effects of EPO on CS toxicity may involve changes in renal phospholipid fatty acid profiles.

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The use of the immunosuppressive drug cyclosporine A (CS) following organ transplantation in humans is often accompanied by both elevations in systemic blood pressure (1–6) and renal arteriolar resistance, and reductions in renal plasma flow and glomerular filtration rate (7–11). While it has been difficult to reproduce this combination of hypertension and nephrotoxicity by administering CS in animal models, it has recently been demonstrated in the borderline hypertensive (BHR) rat using moderate doses of CS (12).

The mechanisms by which CS administration causes hypertension and nephrotoxicity are not fully understood, and it is not known whether CS-induced hypertension is secondary to, or independent of, CS nephrotoxicity. The alteration of renovascular eicosanoid production by CS is one of the postulated mechanisms through which CS may exert its cardiovascular and renal actions. CS has been shown to enhance production of thromboxane A_2 and prostaglandin E_2, as well as to alter prostacyclin synthesis (13–16). Acute CS nephrotoxicity has been reversed by the administration of both thromboxane synthetase inhibitors (17,18) and a prostaglandin E_1 analogue (19).

The ability of dietary fish oils (high in eicosapentaenoic acid, 20:5n-3) and evening primrose oil (EPO; high in γ-linolenic acid, 18:3n-6) to reduce CS-related nephrotoxicity and hypertension may also involve alterations in renal eicosanoid metabolism (12,20,21). High levels of dietary eicosapentaenoic acid lead to a replacement of arachidonic acid (20:4n-6), the precursor of the 2-series prostaglandins, with eicosapentaenoic acid in tissue phospholipids (PL), reducing the amount of substrate available for arachidonic acid-derived eicosanoid synthesis (22,23). The endogenous synthesis of n-3 and n-6 polyunsaturated fatty acids has several rate-limiting steps, namely the Δ6 and Δ5 desaturases which convert 18:2n-6 to 18:3n-6 and 20:3n-6 to 20:4n-6, respectively. As the activities of these enzymes may be affected by certain physiological and/or pathological conditions (24), bypassing these rate-limiting steps through dietary supplementation with γ-linolenic acid, which increases tissue PL levels of its metabolite, dihomo-γ-linolenic acid (the direct precursor for prostaglandin E_1) (25–27), may potentially alter the balance of 1-series and 2-series eicosanoids. It is also possible that CS may alter tissue fatty acid composition and/or metabolism, thereby altering the amount of substrate available for eicosanoid production, and that dietary γ-linolenic acid attenuates CS toxicity by normalizing tissue fatty acid composition.

The purpose of the present study was to examine the effects of (i) CS and EPO on tissue fatty acid composition and (ii) CS administration on the activities of liver microsomal Δ6, Δ5 and Δ9 desaturase enzymes in the genetically BHR.

MATERIALS AND METHODS

Animals. Male BHR, the F_1 offspring of the cross between male Wistar Kyoto and female spontaneously hypertensive rats, were used in these studies. Animals were bred at the University of Waterloo animal facility (21 ± 1°C, 12L/12D) and weaned at 24 d of age onto chow (No. 5001; Ralston Purina, St. Louis, MO) and tap water *ad libitum.*

Protocol. At 36 d of age, rats were randomly assigned into groups (n = 4/group) receiving chow supplemented with 10% (total calories) of either safflower oil (SAF, 2 groups), high in linoleic acid (62% 18:2n-6) or EPO (Efamol Ltd., Guildford, United Kingdom), high in linoleic and γ-linolenic acids (72% 18:2n-6 and 9% 18:3n-6) for 5 wk. One week after being placed on the test diets, one SAF-fed group and the EPO-fed group were administered CS (Sandoz Canada, Dorval, Quebec) at a dose of 10 mg/kg•d p.o. in 0.1 mL olive oil vehicle for 4 wk. The second SAF-fed group received a CS placebo (PLAC; Sandoz Canada, Dorval, Quebec) in 0.1 mL olive oil. All animals were treated

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Abbreviations: BHR, borderline hypertensive rat; CE, cholesteryl ester; CS, cyclosporine A; EPO, evening primrose oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PLAC, CS placebo; PS, phosphatidylserine; SAF, safflower oil; TG, triglyceride.

once daily between 0930–1030 h. As CS in high doses has been reported to reduce body weight gain in experimental animals, the groups receiving CS/EPO (CS treatment and EPO-supplemented diet) and PLAC/SAF (PLAC treatment and SAF-supplemented diet) were pair-fed to the CS/SAF group (CS treatment and SAF-supplemented diet), which was fed *ad libitum*. At the end of the treatment period, liver, kidneys, heart and brain were removed from halothane-anesthetized animals, frozen in liquid nitrogen and stored at −85°C for tissue fatty acid analaysis.

In a second study, eleven male BHR of 36 d of age were placed on SAF-supplemented chow diets and administered either PLAC (n = 5) or CY (n = 6) for 4 wk, as described above. At the end of the treatment period, systolic blood pressure measurements were made on conscious animals, using the tail-cuff method (28). Livers were then removed as described above, quickly rinsed in cold saline (0.9% NaCl) and frozen in liquid nitrogen for later isolation of microsomes and measurement of *in vitro* microsomal Δ9, Δ6 and Δ5 desaturase activities.

Fatty acid analysis. Lipids were extracted from approximately 1.5 g tissue with chloroform/methanol (2:1, vol/vol), as previously described (28). Total PL, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine/phosphatidylinositol (PS/PI), triglyceride (TG) and cholesteryl ester (CE, liver only) fractions were then separated by thin-layer chromatography and subsequently transmethylated with BF_3-methanol (29). The fatty acid methyl esters were analyzed on a Perkin Elmer gas chromatograph (model 8420; Norwalk, CT) equipped with a flame-ionization detector and a 30-m capillary column (Supelcowax 10; Supelco, Bellefonte, PA). The temperature was programmed to be held for 2 min at 180°C, followed by a 2°C/min increase to 220°C, and a 2 min hold at 220°C. Fatty acids were identified by comparison of their retention time with those of known standards.

Measurement of desaturase activity. Microsomes were prepared from 5 g liver tissue within 24 h of freezing, as previously described (30), and stored at −80°C until analysis. The activities of Δ9, Δ6 and Δ5 desaturases were determined by measuring the conversion of [1-^{14}C]-palmitic acid (16:0) to [1-^{14}C]palmitoleic acid (16:1n-7), of [1-^{14}C]linoleic acid to [1-^{14}C]γ-linolenic acid and of [1-^{14}C]dihomo-γ-linolenic acid to [1-^{14}C]arachidonic acid, respectively. Reactions were started by adding microsomal protein (5 mg) to pre-incubated tubes containing 25 μCi of the substrate fatty acid at a final concentration of 53.3 μM in 1.5 mL of the incubation solution, containing ATP (1.30 mM), NADH (0.87 mM) and coenzyme A (0.06 mM) as previously described (31). The tubes were vortexed vigorously and, after 15 min incubation in a shaking water bath (37°C), the reactions were stopped by the addition of 2 mL 10% (wt/vol) KOH in ethanol. Lipids in the incubation mixture were saponified at 80°C for 45 min under N_2. After acidification, fatty acids were extracted with hexane. Radiolabelled fatty acids were analyzed according to the method of Narce *et al.* (32). Analyses were carried out in a high-performance liquid chromatograph (model 510 pump and 712 WISP autosampler; Waters, Milford, MA) equipped with a variable wavelength UV-vis monitor (model 450, set at 205 nm) and a radioisotope detector (model 171; Beckman, Fullerton, CA). Fatty acids were separated isocratically with acetonitrile/water (95:5, vol/vol) at a flow rate of 1 mL/min and were identified by comparison with authentic standards.

Statistics. Fatty acid composition data across the three treatment groups were analyzed in each tissue using a one-way analysis of variance. Where a significance of $P < 0.05$ was achieved, specific group differences were evaluated using the Student Newman-Keuls test. Blood pressure and desaturase activity data from the two treatment groups were analyzed using the Student's *t*-test.

RESULTS

Body weight gain did not significantly differ among the treatment groups in this study (data not shown).

Effects of CS and diet on tissue fatty acid composition. CS supplementation of SAF-fed animals did not affect fatty acid composition (% total fatty acids) of the hepatic total PL, TG, PS/PI, PC or the CE fraction (data not shown). However, EPO feeding of CS-treated animals affected the levels of several fatty acids independent of CS. Specifically, EPO feeding increased 18:1n-7 ($P < 0.001$) and decreased 18:3n-3 ($P < 0.05$) in the total PL fraction, decreased 18:0 ($P < 0.01$) in the PC fraction, and decreased 18:0 ($P < 0.01$), 18:2n-6 ($P < 0.001$), and 22:5n-3 ($P < 0.05$) in the CE fraction *vs.* the CS/SAF group (data not shown). In contrast, in the PE fraction (Table 1), administration of CS to SAF-fed rats significantly increased 16:0 ($P < 0.05$), 18:1n-9 ($P < 0.01$), and 18:2n-6 ($P < 0.05$) *vs.* PLAC. These changes were reversed by the presence of dietary EPO with CS. EPO-feeding also independently decreased 18:0 and 20:4n-6 ($P < 0.05$) *vs.* CS/SAF in this fraction.

In the kidney, CS administration to SAF-fed animals significantly altered fatty acid levels in several lipid fractions *vs.* the PLAC/SAF group (Table 2). Specifically, CS increased 18:2 ($P < 0.05$) in the TG fraction, increased 16:0 ($P < 0.001$) and decreased 20:4n-6 ($P < 0.01$) in the PS/PI fraction, and decreased 18:0 ($P < 0.05$) and 18:1n-9 ($P < 0.01$) in the PC fraction. All of these changes were reversed by feeding EPO to CS-treated animals, restoring their

TABLE 1

Composition of Major Saturated, n-9, n-6 and n-3 Fatty Acids (% total fatty acids; $\bar{X} \pm SE$, n = 4/group) of Liver Phosphatidylethanolamine in Male Borderline Hypertensive Rats Fed Either SAF or EPO and Treated with Either PLAC or CS

Fatty acid	PLAC/SAF	CS/SAF	CS/EPO
16:0	9.1 ± 1.9	13.6 ± 1.7[a]	5.5 ± 1.4[c]
18:0	28.5 ± 1.7	29.6 ± 3.7	15.4 ± 5.0[a,c]
18:1n-9	3.9 ± 0.4	6.5 ± 0.6[b]	1.5 ± 0.5[a,e]
18:1n-7	0.8 ± 0.5	1.5 ± 0.5	1.7 ± 0.8
18:2n-6	7.9 ± 1.7	13.5 ± 1.6[a]	3.9 ± 1.3[d]
18:3n-3	—	—	0.2 ± 0.2
20:3n-6	0.7 ± 0.5	0.8 ± 0.3	0.7 ± 0.3
20:4n-6	19.7 ± 0.7	19.7 ± 1.1	12.0 ± 2.4[a,c]
20:5n-3	0.2 ± 0.2	0.3 ± 0.2	3.4 ± 3.2
22:4n-6	1.9 ± 1.3	2.4 ± 1.1	2.5 ± 2.3
22:5n-3	1.3 ± 0.5	0.5 ± 0.3	2.4 ± 1.3
22:6n-3	2.7 ± 0.3	3.3 ± 0.3	2.1 ± 1.2

[a]$P < 0.05$ *vs.* PLAC/SAF, [b]$P < 0.01$ *vs.* PLAC/SAF, [c]$P < 0.05$ *vs.* CS/SAF, [d]$P < 0.01$ *vs.* CS/SAF, [e]$P < 0.001$ *vs.* CS/SAF. Abbreviations: SAF, safflower oil; EPO, evening primrose oil; PLAC, CS placebo; CS, cyclosporine A.

TABLE 2

Composition of Major Saturated and Unsaturated Fatty Acids (% total fatty acids; $\bar{X} \pm SE$, n = 4/group) of Kidney Phosphatidylethanolamine (PE), Phosphatidylserine/Phosphatidylinositol (PS/PI) and Phosphatidylcholine (PC) in Male Borderline Hypertensive Rats Fed Either SAF or EPO and Treated with Either PLAC or CS

	16:0	18:0	18:1n-9	18:1n-7	18:2n-6	18:3n-3	20:3n-6	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3
Kidney TG												
PLAC/SAF	24.4 ± 0.7	4.1 ± 0.5	22.5 ± 0.8	1.5 ± 0.1	21.7 ± 1.5	0.4 ± 0.04	0.1 ± 0.01	0.3 ± 0.1	0.1 ± 0.03	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.06
CS/SAF	20.9 ± 0.7	4.9 ± 0.2	21.1 ± 0.7	1.7 ± 0.2	31.2 ± 2.4[a]	0.5 ± 0.04	0.2 ± 0.02	0.8 ± 0.2	0.1 ± 0.02	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
CS/EPO	21.4 ± 2.7	4.0 ± 0.6	18.4 ± 1.6	1.7 ± 0.2	20.7 ± 3.0[d]	0.6 ± 0.3	0.4 ± 0.2[a]	1.1 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.6 ± 0.1[a]
Kidney PS/PI												
PLAC/SAF	12.3 ± 3.7	20.9 ± 1.7	7.9 ± 2.9	2.1 ± 0.2	6.3 ± 4.5	—	0.2 ± 0.2	15.3 ± 2.8	0.6 ± 0.2	0.3 ± 0.2	—	0.4 ± 0.2
CS/SAF	40.6 ± 2.9[c]	16.3 ± 1.2	7.7 ± 0.3	2.6 ± 0.3	8.4 ± 0.5	0.4 ± 0.4	0.3 ± 0.1	4.8 ± 0.9[b]	0.2 ± 0.02	0.1 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
CS/EPO	8.5 ± 2.2[f]	26.9 ± 4.3[d]	4.8 ± 0.6	1.4 ± 0.3	2.8 ± 1.2	—	0.6 ± 0.2	17.6 ± 2.0[e]	0.3 ± 0.2	0.9 ± 0.1[d]	0.6 ± 0.4	0.7 ± 0.02
Kidney PC												
PLAC/SAF	44.8 ± 1.6	12.0 ± 0.5	8.4 ± 0.2	2.5 ± 0.1	9.9 ± 1.3	—	0.3 ± 0.1	3.1 ± 0.9	0.2 ± 0.03	0.2 ± 0.1	1.3 ± 0.1	0.4 ± 0.1
CS/SAF	33.9 ± 2.5	7.6 ± 0.7[a]	5.7 ± 0.2[b]	2.1 ± 0.1	12.6 ± 1.4	—	0.5 ± 0.1	8.2 ± 1.3	—	—	1.6 ± 0.1	0.5 ± 0.05
CS/EPO	40.1 ± 4.8	13.0 ± 1.9[d]	7.0 ± 0.7	2.6 ± 0.4	8.4 ± 2.3	—	0.5 ± 0.3	7.8 ± 5.8	0.1 ± 0.1	0.1 ± 0.03	1.1 ± 0.3	0.6 ± 0.5

[a]$P < 0.05$ *vs.* PLAC/SAF, [b]$P < 0.01$ *vs.* PLAC/SAF, [c]$P < 0.001$ *vs.* PLAC/SAF, [d]$P < 0.05$ *vs.* CS/SAF, [e]$P < 0.01$ *vs.* CS/SAF, [f]$P < 0.001$ *vs.* CS/SAF. TG, triglycerides. See Table 1 for other abbreviations.

TABLE 3

Composition of Major Saturated and Unsaturated Fatty Acids (% total fatty acids; $\bar{X} \pm SE$, n = 4/group) of Heart Total Phospholipids (PL), Phosphatidylethanolamine (PE), Phosphatidylserine/Phosphatidylinositol (PS/PI) and Phosphatidylcholine (PC) in Male Borderline Hypertensive Rats Fed Either SAF or EPO and Treated with Either PLAC or CS

	16:0	18:0	18:1n-9	18:1n-7	18:2n-6	18:3n-3	20:3n-6	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3
Heart PL												
PLAC/SAF	10.8 ± 0.8	22.0 ± 1.2	2.9 ± 0.1	3.0 ± 0.1	16.9 ± 1.0	0.3 ± 0.3	0.4 ± 0.1	17.1 ± 1.1	0.5 ± 0.2	0.7 ± 0.1	1.5 ± 0.1	8.9 ± 0.7
CS/SAF	12.5 ± 0.3	23.6 ± 1.0	3.3 ± 0.2	3.3 ± 0.1	15.0 ± 0.5	0.1 ± 0.02	0.3 ± 0.01	20.2 ± 0.6[a]	0.2 ± 0.03	0.7 ± 0.03	1.5 ± 0.1	10.0 ± 0.2
CS/EPO	11.2 ± 0.6	20.5 ± 1.5	2.7 ± 0.02[e]	3.2 ± 0.1	15.5 ± 1.5	0.4 ± 0.2	0.5 ± 0.1	20.4 ± 1.0[a]	0.3 ± 0.1	0.8 ± 0.1	1.5 ± 0.04	8.8 ± 0.4
Heart PE												
PLAC/SAF	10.2 ± 2.5	24.8 ± 4.7	7.0 ± 3.7	2.7 ± 0.1	18.1 ± 2.8	01 ± 0.1	—	15.1 ± 3.9	0.4 ± 0.3	3.2 ± 1.0	1.4 ± 0.3	6.5 ± 1.9
CS/SAF	9.1 ± 0.5	34.0 ± 3.6	3.5 ± 0.2[a]	2.5 ± 0.1[b]	8.5 ± 0.6	—	—	20.9 ± 1.1[a]	0.1 ± 0.1	1.4 ± 0.3	1.9 ± 0.1	11.5 ± 1.7[a]
CS/EPO	9.8 ± 1.0	28.0 ± 2.5	5.8 ± 1.6	2.9 ± 0.5	15.0 ± 4.1	—	0.1 ± 0.1	17.6 ± 1.7	0.1 ± 0.1	3.9 ± 2.3	1.9 ± 0.3	8.1 ± 3.0
Heart PS/PI												
PLAC/SAF	6.8 ± 2.4	47.3 ± 2.0	6.3 ± 1.3	0.6 ± 0.6	12.2 ± 0.8	—	—	23.5 ± 1.8	—	—	—	1.8 ± 1.0
CS/SAF	9.8 ± 1.5	33.6 ± 2.7[c]	3.4 ± 0.2[b]	2.5 ± 0.2[b]	10.5 ± 0.7	—	0.2 ± 0.1	28.8 ± 0.7[a]	—	1.0 ± 0.4	1.8 ± 0.2	7.5 ± 13.2[c]
Heart PC												
PLAC/SAF	14.2 ± 0.8	24.1 ± 1.3	3.3 ± 0.04	4.2 ± 0.4	15.2 ± 0.8	0.3 ± 0.2	0.4 ± 0.1	19.3 ± 1.1	0.5 ± 0.2	0.6 ± 0.1	1.2 ± 0.1	2.9 ± 0.9
CS/SAF	25.4 ± 1.0[c]	24.2 ± 1.2	4.8 ± 0.4[a]	6.1 ± 0.2[a]	13.9 ± 1.8	—	0.2 ± 0.1	8.6 ± 3.6[a]	—	1.1 ± 0.3	0.8 ± 0.1	3.4 ± 0.5
CS/EPO	23.5 ± 0.8[c]	26.0 ± 1.8	3.6 ± 0.3[d]	6.6 ± 0.6[b]	10.3 ± 1.5	—	0.3 ± 0.2	7.7 ± 2.1[b]	—	1.6 ± 0.4	0.3 ± 0.2[b]	2.8 ± 0.7

[a]$P < 0.05$ *vs.* PLAC/SAF, [b]$P < 0.01$ *vs.* PLAC/SAF, [c]$P < 0.001$ *vs.* PLAC/SAF, [d]$P < 0.05$ *vs.* CS/SAF. [e]$P < 0.001$ *vs.* CF/SAF. See Table 1 for abbreviations.

levels to those of the PLAC/SAF group (Table 2). EPO feeding also independently increased 20:3n-6 ($P < 0.05$) in total PL (data not shown) and 20:3n-6 and 22:6n-3 ($P < 0.05$) in kidney TG (Table 2), and increased 18:0 and 22:4n-6 ($P < 0.05$) in the PS/PI fraction *vs.* CS/SAF (data not shown).

In cardiac tissue, CS administration to SAF-fed rats increased 20:4n-6 ($P < 0.05$) *vs.* PLAC/SAF in the total PL, decreased 18:1n-9 ($P < 0.05$) and increased 20:4n-6 and 22:6n-3 ($P < 0.05$) in the PE fraction. It also increased 16:0 ($P < 0.001$), 18:1n-9 and 18:1n-7 ($P < 0.05$) and decreased 20:4n-6 ($P < 0.05$) in the PC fraction (Table 3). CS administration also decreased 18:0 ($P < 0.001$) and 18:1n-9 ($P < 0.01$) and increased 18:1n-7 ($P < 0.01$), 20:4n-6 ($P < 0.05$) and 22:6n-3 ($P < 0.001$) *vs.* PLAC in the PS/PI fraction. Due to a loss of samples from the CS/EPO group of this fraction, only the SAF-fed groups are reported. EPO-feeding of CS-treated animals did not reverse the effects of CS administration (with the exception of 18:1n-9 in the PC fraction). EPO independently decreased 18:1n-9 ($P < 0.01$) and increased 20:3n-6 ($P < 0.05$) in the total PL and TG, respectively (data not shown).

In the brain, CS administration significantly increased 20:5n-3 ($P < 0.05$) in brain TG, and increased 18:0, 18:1n-9 ($P < 0.01$), and 18:1n-7 ($P < 0.05$) in the PE fraction *vs.* PLAC/SAF (Table 4). EPO feeding to CS-treated animals reversed the CS effects on 20:5n-3 in the TG fraction, and 18:0 and 18:1n-9 in the PE fraction. EPO feeding also increased 16:0 ($P < 0.01$) in the TG fraction. EPO feeding had independent effects on brain fatty acid composition in other fractions, decreasing 18:0 ($P < 0.05$) *vs.* CS/SAF in total PL, and decreasing 20:3n-6, 20:4n-6, 22:4n-6 ($P < 0.05$), and 22:6n-3 ($P < 0.001$) in the PC fraction (data not shown).

Blood pressure in PLAC and CS-treated BHR. After 4 wk of treatment, conscious resting blood pressure in SAF-fed rats was higher in the group receiving CS than in those receiving PLAC (152 ± 2 mm Hg *vs.* 134 ± 1 mm Hg, $P < 0.001$).

Liver desaturase activities. The effects of CS on liver microsomal Δ9, Δ6 and Δ5 desaturase activities are shown in Figure 1. CS significantly elevated the activity of Δ9

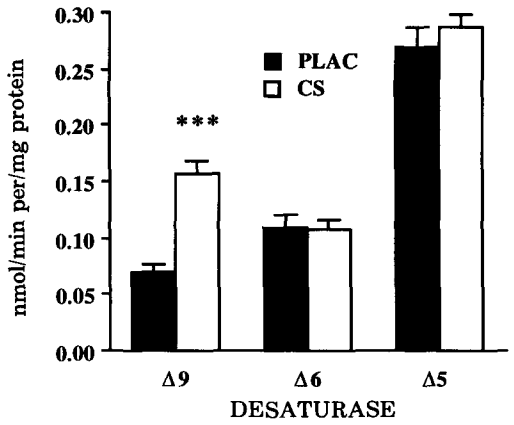

FIG. 1. ***In vitro*** **activities (nmol/min•mg protein) of liver microsomal Δ9, Δ6 and Δ5 desaturase enzymes following 4 wk of treatment with either cyclosporine A (CS) (10 mg/kg•d p.o.) or placebo (PLAC) (n = 5/group) in borderline hypertensive rats fed safflower oil. Values are $\bar{X}$ ± SE.**

TABLE 4

Composition of Major Saturated and Unsaturated Fatty Acids (% total fatty acids; $\bar{X}$ ± SE, n = 4/group) of Brain Triglycerides (TG) and Phosphatidylethanolamine (PE) in Male Borderline Hypertensive Rats Fed Either SAF or EPO and Treated with Either PLAC or CS

	16:0	16:1n-7	18:0	18:1n-9	18:1n-7	18:2n-6	20:3n-6	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3
Brain TG												
PLAC/SAF	17.5 ± 0.7	1.1 ± 0.6	23.4 ± 0.6	15.1 ± 1.2	3.6 ± 0.2	2.1 ± 0.8	0.1 ± 0.1	8.3 ± 1.3	0.1 ± 0.1	3.2 ± 1.0	0.4 ± 0.3	7.6 ± 1.6
CS/SAF	14.5 ± 2.1	–	20.0 ± 2.1	14.2 ± 1.6	3.3 ± 0.3	5.2 ± 1.5	0.1 ± 0.1	8.0 ± 0.6	4.5 ± 1.1[a]	4.2 ± 1.1	0.9 ± 0.4	7.0 ± 1.9
CS/EPO	23.0 ± 1.5[b,c]	0.2 ± 0.2	19.6 ± 1.0	15.6 ± 2.8	3.1 ± 1.0	3.6 ± 0.4	0.1 ± 0.1	12.5 ± 1.5	0.1 ± 0.1[c]	1.3 ± 0.9	0.2 ± 0.2	6.1 ± 1.1
Brain PE												
PLAC/SAF	5.2 ± 0.3	0.4 ± 0.03	18.3 ± 0.5	14.1 ± 0.6	2.2 ± 0.1	0.5 ± 0.02	0.5 ± 0.04	11.6 ± 0.3	0.1 ± 0.1	3.8 ± 0.2	0.2 ± 0.1	17.4 ± 0.4
CS/SAF	5.6 ± 0.7	0.3 ± 0.2	29.1 ± 2.4[b]	18.5 ± 0.2[b]	2.8 ± 0.2[a]	0.3 ± 0.2	0.6 ± 0.01	9.4 ± 0.9	–	3.5 ± 0.3	0.1 ± 0.1	17.8 ± 0.7
CS/EPO	4.7 ± 0.3	0.3 ± 0.1	19.2 ± 1.1[c]	13.4 ± 0.8[c]	2.3 ± 0.2	0.2 ± 0.1[a]	0.5 ± 0.2	10.1 ± 0.6	–	14.5 ± 9.9	0.2 ± 0.1	14.3 ± 1.5

[a]$P < 0.05$ *vs.* PLAC/SAF, [b]$P < 0.01$ *vs.* PLAC/SAF, [c]$P < 0.01$ *vs.* CS/SAF. See Table 1 for abbreviations.

desaturase *vs.* PLAC by 124%, but had no effect on the activities of either Δ6 or Δ5 desaturase.

DISCUSSION

The use of CS as an immunosuppressant in humans has frequently been reported to be accompanied by toxic renal, cardiovascular and psychological side effects, which can limit its usefulness in the clinical setting (33). These undesirable side effects have been reported to be prevented by dietary supplementation with either eicosapentaenoic acid (20:5n-3, in the form of fish oil) or γ-linolenic acid (18:3n-6, in the form of EPO) (20,21). As these fatty acids may alter endogenous eicosanoid metabolism, their attenuation of the side effects of CS may involve alterations of tissue eicosanoid metabolism (Scholey and Mills, unpublished observation).

The present study demonstrated that, in the BHR, chronic administration of CS was accompanied by significant changes in the fatty acid composition of PL in a variety of tissues, most often affecting the 16 and 18 carbon fatty acids.

A second finding of the study was that the substitution of EPO for SAF in the diet of animals treated with CS led to a normalization of PL fatty acid composition in almost every case in the liver and kidney, so that the fatty acid levels were similar to those of the PLAC/SAF treatment group. In addition to reversing the changes in PL fatty acid composition brought about through CS administration, dietary EPO itself appeared to affect fatty acid composition in liver, heart and kidney. Of particular interest in this regard are (i) the increase in 20:3n-6, a precursor for prostaglandin E_1 and other eicosanoids, seen in the TG fractions of kidney and heart and in the PL of kidney and liver, and (ii) the decrease in 20:4n-6 seen in liver PE. TG stores of the renal medulla have been shown to be a source of fatty acids for prostaglandin synthesis (34). Increased 20:3n-6 levels in this fraction may, through their role as eicosanoid substrates, shift the balance of prostanoid production in the renal medulla. While not conclusive, the present results are supportive of the theory of fatty acid and eicosanoid involvement in CS-induced nephrotoxicity as well as a renal component in CS-induced hypertension. The normalization of renal PL fatty acid composition by dietary EPO may also contribute to its ability to alleviate CS-induced nephrotoxicity and hypertension.

The level of Δ6 desaturase activity observed in the prsent study is similar to that previously reported in Wistar rats of a similar age at a similar time of the year (35). The absence of an effect of CS on the *in vitro* activity of liver microsomal Δ6 and Δ5 desaturases in the present study suggests that CS-induced changes in tissue fatty acid composition do not involve alterations of the rate-limiting steps in essential fatty acid metabolism. This is consistent with an absence of changes in Δ6 and Δ5 desaturated n-6 and n-3 fatty acids in the liver of CS-treated rats. The activity of Δ9 desaturase that we observed in the control, PLAC, group was lower than that previously reported for Wistar rats of a similar age at a similar time of the year (35). Whether this discrepancy between the two studies is due to differences in the strains of rat used is unknown. However, the activity observed in the present study is within the range previously observed across the seasons of the year (35). Nonetheless, despite a significant increase in the *in vitro* activity of liver microsomal Δ9 desaturase caused by CS, this was not reflected in the fatty acid composition of total lipid extracts from livers from CS-treated rats. This suggests that CS-induced changes in tissue lipids may involve other aspects of fatty acid metabolism, *e.g.*, incorporation into and/or removal from tissue PL.

It has been suggested that CS nephrotoxicity may reflect a renal transmembrane signalling disorder and that this defect in signal transduction may result in an enhanced vasoconstriction in the whole kidney (36). CS administration has been observed to increase both basal and stimulated cytosolic calcium levels in both mesangial and vascular smooth muscle cells in culture (37-39), a process that involves the activation of phospholipase C and a subsequent inositol triphosphate-mediated release of calcium from intracellular stores, followed by a calcium influx from the external environment (40,41). Furthermore, CS has been shown to inhibit the release of 20:4n-6 from the PL fraction in isolated glomeruli and endothelial cell preparations (35,42,43), an effect which may reduce the avaiability of vasodilatory prostaglandins. It is possible that the alteration of renal PS/PI fatty acid composition following CS administration may contribute to this process, and that the normalization of the serine/inositol PL fatty acid composition, as well as the increase in TG 20:3n-6, by EPO may partially explain its attenuation of CS nephrotoxicity and hypertension.

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Effects of Dietary Protein and Cholesterol on Phosphatidylcholine and Phosphatidylethanolamine Molecular Species in Mouse Liver

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The present study examined the effects of two atherogenic factors, animal protein and cholesterol, on the distribution of fatty acids and the molecular species of major liver phospholipids in mice. Weanling mice were fed a semisynthetic diet supplemented with either casein or soy protein (20%, w/w) in the presence or absence of 0.5% cholesterol for 4 wk. Results from mouse liver showed that animal protein and, more so, dietary cholesterol modified the fatty acid profiles of the phospholipids. Animal protein had no significant effect on the concentration of lipids, but it altered the relative distribution and fatty acid profiles of the phospholipids, phosphatidylcholine and phosphatidylethanolamine. Dietary cholesterol, on the other hand, significantly increased the concentration of liver lipids, but it did not alter the relative distribution of phosphatidylcholine and phosphatidylethanolamine. In cholesterol-fed mice, the proportions of molecular species containing 18:2n-6 were increased, whereas those containing 20:4n-6 were decreased, indicating that dietary cholesterol suppressed linoleic acid metabolism. Since cholesterol feeding selectively decreased the ratio of 18:0/20:4n-6 in phosphatidylcholine, whereas it increased the 18:0/18:2n-6 ratio in phosphatidylethanolamine, this finding suggests that dietary cholesterol may affect the incorporation of fatty acids but not the rate of synthesis of phosphatidylcholine and phosphatidylethanolamine.
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In several experimental animals, dietary animal protein and cholesterol (CHOL) are both known to be atherogenic, but they influence fatty acid metabolism differently (1). In rats, dietary supplementation with animal protein, in comparison with vegetable protein, increases the activity of Δ6 desaturase in liver microsomes (2-6), and the ratio of 20:4n-6/18:2n-6 in liver phospholipids (PL) (2-8). CHOL feeding, on the other hand, suppresses the metabolism of n-6 fatty acids (9-12). Since the effective conversion of 18:2n-6 to long-chain n-6 fatty acids is essential to the CHOL-lowering effect of dietary polyunsaturated fatty acids (PUFA, mainly 18:2n-6) (13-15), it is paradoxical that these two atherogenic factors (animal protein and CHOL) affect the metabolism of linoleic acid in such a divergent fashion.

The interaction of CHOL, protein and PUFA has previously been examined in rats. Since rats, in comparison with other animal species, have active linoleic acid metabolism, it is of interest to examine whether the above-mentioned interactions also take place in other species with less active linoleic acid metabolism.

It is known that enzymes that are responsible for linoleic acid metabolism are associated with microsomal membranes and that PL constitute the major components of membranes. We examined in the present study whether dietary CHOL and protein would affect the molecular species of two major PL fractions in liver microsomes in C57BL/6 mice. This animal species, like the human, has a relatively low rate of linoleic acid metabolism (16) and is susceptible to CHOL feeding (17).

MATERIALS AND METHODS

Chemicals. Cold-pressed safflower oil (containing 6.8%, 16:0; 2.6%, 18:0; 12.3%, 18:1n-9; and 77.1%, 18:2n-6) and linseed oil (containing 5%, 16:0; 3.6%, 18:0; 23.3%, 18:1n-9; 14.2%, 18:2n-6; and 53.5%, 18:3n-3) were purchased from a local health food store. CHOL and phospholipase C (*Bacillus cereus*, Type III) were supplied by Sigma Chemical Co. (St. Louis, MO). Highly purified casein (CAS, 89% protein and 0.06% lipids), soy protein isolate (SOY, containing 89% protein and 0.4% lipids), vitamin mix (AIN-76), mineral mix (AIN-76), cellulose and corn starch were obtained from Teklad Test Diets (Madison, WI). All solvents were of high-performance liquid chromatography (HPLC) grade and were supplied by Fisher Scientific (Nepean, Ontario, Canada).

Animals and diets. Twenty weanling male C57BL/6 mice (3-week-old), purchased from Charles River Breeding Laboratories (Montreal, Quebec, Canada), were given free access to a CHOL-free diet consisting (by weight percent) of CAS, 21.8; fat mixture, 2.0; cellulose, 5.4; DL-methionine, 0.3; mineral mix, 3.8; vitamin mix, 1.1; calcium carbonate, 0.08; and sucrose, 65.5. After 7 d of acclimatization, the mice were divided into four groups (five animals each), which received different dietary proteins: CAS or SOY, with or without an additional 0.5% (w/w) CHOL. The animals were maintained *ad libitum* on the assigned diets for 2 wk. The details of the diets, prepared according to the formula recommended by the American Institute of Nutrition (18), are shown in Table 1. The dietary fat was a 72:28 (w/w) mixture of safflower oil and linseed oil to give an n-6/n-3 ratio of 3.9 (Table 1). At the end of the study, mice were lightly anesthetized with diethyl ether and sacrificed by exsanguination through cardiac puncture. The liver was excised immediately and frozen at −80°C prior to extraction.

Lipid analysis. Total liver lipids were extracted by the method of Folch *et al.* (19). The concentrations of liver CHOL were measured by gas-liquid chromatography (GLC) (9), and liver PL were quantified by phosphorus content following the method of Rouser *et al.* (20). Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), sphingomyelin and lysophospholipids were separated by thin-layer chromatography using chloroform/ethanol/water/triethylamine (4:5:1:5, by vol) as the developing solvent. The

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Abbreviations: CAS, casein; CHOL, cholesterol; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SOY, soy protein isolate.

TABLE 1

Composition of Diet[a]

Ingredient	CAS	CAS + CHOL	SOY	SOY + CHOL
			(wt%)	
Casein	20.0	20.0	—[c]	—
Soy protein isolate	—	—	20.0	20.0
Fat[b]	5.0	5.0	5.0	5.0
Corn starch	15.0	15.0	15.0	15.0
Cellulose	5.0	5.0	5.0	5.0
Mineral mix (AIN-76)	3.5	3.5	3.5	3.5
Vitamin mix (AIN-76)	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	
Cholesterol	—	0.5	—	0.5
Sucrose	50.3	49.8	50.3	49.8

[a]Abbreviations: CAS, casein; SOY, soy protein isolate; CHOL, cholesterol.
[b]A mixture of safflower oil and linseed oil (72:28, w/w), containing 6.3%, 16:0; 2.8%, 18:0; 15.3%, 18:1n-9; 59.7%, 18:2n-6; and 15.4%, 18:3n-3. The n-6/n-3 ratio was 3.9.
[c]—, not added in the diet.

distribution of PL classes was determined by their phosphorus content (19). The fatty acid composition of liver PC and PE was determined by GLC after transmethylation with BF_3 methanol (21).

For separation of molecular species, approximately 1 mg of either PC or PE in diethyl ether (2 mL) was incubated for 3 h at room temperature with 1 mg of phospholipase C (*Bacillus cereus,* Type III) in 2 mL of 0.5 M tris buffer (pH 7.5) and 2 mM calcium chloride (22). The resultant mixture of diacylglycerols was acetylated with acetic anhydride/pyridine (1:2, vol/vol) at 37°C for 1 h (23). The solution was taken to dryness, and the residue was redissolved in acetonitrile. The molecular species were separated by reverse-phase HPLC on a Zorbax ODS column (250 mm × 4.6 mm, i.d.) (DuPont Co., Wilmington, DE), using an isocratic solvent system of acetonitrile/2-propanol/methyl *t*-butyl ether/water (72:18:8:2, by vol) at a flow rate of 0.5 mL/min)24). For identification of molecular species in each fraction, the fractions detected at 205 nm with a variable wavelength detector (Diode Array Detector Module 168; Beckman, Palo Alto, CA) were collected, and the fatty acid composition was determined by GLC (21). The distribution of peaks was assessed using a mass (light-scattering) detector (ACS Model 740/14; Applied Chromatography Systems, Macclesfield, United Kingdom). The detailed distribution and identification of PL molecular species (in the form of acetylated diacylglycerol) co-eluted in the same fraction were determined based on a computer program similar to that described previously (25). Briefly, it is based on random probability using the fatty acid composition of the purified PL fraction and on the retention times, which reflect the equivalent chain lengths. Figure 1 shows typical HPLC chromatograms.

Statistical analysis. Data were expressed as mean ± SD of five animals. Statistical analyses were performed using analysis of variance through a computer statistical system, SYSTAT (26). The 2 × 2 factorial analysis determining the protein and CHOL effects and interactions

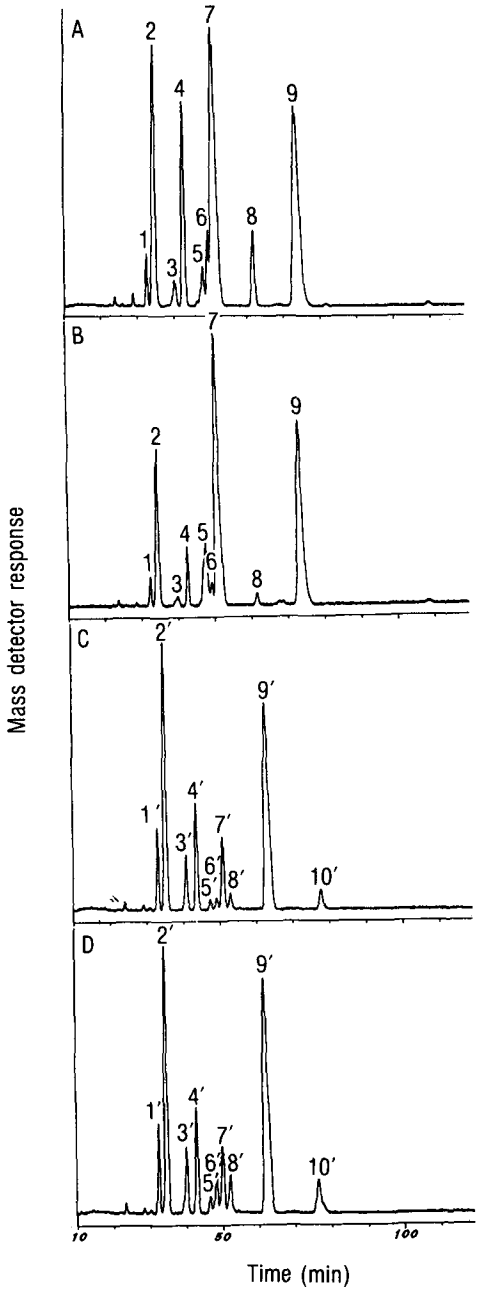

FIG. 1. Typical high-performance liquid chromatography chromatograms of liver phosphatidylcholine (panels A and B) and phosphatidylethanolamine (panels C and D) molecular species in mice fed a casein diet without (panels A and C) or with (panels B and D) 0.5% cholesterol. In panels A and B, fraction 1 contains 16:0/20:5n-3 and 18:1/22:6n-3; fraction 2 contains 16:0/22:6n-3, 16:1/18:2n-6, 18:2n-6/18:2n-6 and 16:1/20:3n-6; fraction 3 contains 18:1/20:4n-6, 16:0/22:5n-3 and 16:0/18:3n-6; fraction 4 contains 16:0/20:4n-6; fraction 5 contains 18:1/18:2n-6, 18:1/20:3n-6, 16:0/16:1 and 16:1/18:1; fraction 6 contains 18:0/22:6n-3; fraction 7 contains 16:0/18:2n-6 and 16:0/20:3n-6; fraction 8 contains 18:0/20:4n-6; and fraction 9 contains 18:0/18:2n-6, 16:0/18:1 and 18:0/20:3n-6. In panels C and D, fraction 1′ contains 18:1/22:6n-3 and 16:0/20:5n-3; fraction 2′ contains 16:0/22:6n-3; fraction 3′ contains 18:1/20:4n-6 and 16:0/22:5n-3; fraction 4′ contains 16:0/20:4n-6; fraction 5′ contains 18:0/20:5n-3 and 16:0/22:5n-6; fraction 6′ contains 18:1/18:2n-6, 16:0/16:1 and 18:1/20:3n-6; fraction 7′ contains 18:0/22:6n-3; fraction 8′ contains 16:0/18:2n-6, 18:0/18:3n-3, 16:1/18:1 and 16:0/20:3n-6; fraction 9′ contains 18:0/20:4n-6; and fraction 10′ contains 18:0/18:2n-6, 18:0/20:3n-6, and 16:0/18:1.

between dietary protein and CHOL was followed by the least-square mean multiple comparison to identify the dietary group differences.

RESULTS

Body and liver weight. Table 2 shows that there were no significant differences between dietary groups in either the initial or the final body weights, or the relative liver weights at the time of killing. The concentrations of PL and CHOL and the CHOL/PL ratio in liver were not affected by different dietary protein sources but were increased significantly by CHOL supplementation. However, the PL distribution was modulated by both dietary protein and CHOL. CAS, as compared to SOY, increased the proportion of liver PC, whereas it decreased that of PI, independent of CHOL feeding. Dietary CHOL decreased the proportion of PE whereas PS was increased in both protein groups. The PC/PE ratio was higher ($P = 0.016$) in mice fed the CAS diets than in those fed the SOY diets.

Fatty acid profiles of liver PL. In liver, PC and PE represent the two major PL classes. Hence, we have chosen to report only the changes in these two PL fractions. Table 3 shows the fatty acid composition of liver PC. CHOL feeding significantly increased the proportion of 18:2n-6, whereas it decreased those of 20:4n-6 and 22:6n-3. As a result, CHOL feeding depressed significantly the ratios of (18:3n-6 + 20:3n-6 + 20:4n-6)/18:2n-6 and of 20:4n-6/20:3n-6. Dietary protein, on the other hand, had little

TABLE 2

Effects of Dietary Protein and Cholesterol on Body and Liver Weight and Liver Phospholipid and Cholesterol Concentrations in Mice[a]

	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
Body weight (g)							
Initial	16.4 ± 1.4	16.4 ± 1.6	16.4 ± 2.0	15.8 ± 1.9	—[b]	—	—
Final	22.5 ± 2.7	23.8 ± 1.7	22.5 ± 2.9	23.3 ± 1.7	—	—	—
Liver weight (g/100 g body weight)	5.6 ± 0.1	5.7 ± 0.9	5.7 ± 0.5	6.3 ± 0.9	—	—	—
Lipid content (μmol/g liver)							
Phospholipids	32.4 ± 2.2	36.9 ± 1.4	32.3 ± 2.2	37.7 ± 2.4	—	0.001	—
Cholesterol	6.4 ± 0.5	43.0 ± 7.8	5.8 ± 0.9	46.5 ± 13.1	—	0.001	—
CHOL/PL ratio	0.2 ± 0.0	1.2 ± 0.3	0.2 ± 0.0	1.2 ± 0.4	—	0.001	—
PL distribution (mol%)							
PE	25.7 ± 0.8	24.4 ± 0.9	26.2 ± 0.6	24.9 ± 1.0	—	0.004	—
PI	8.6 ± 1.0	9.5 ± 1.1	10.2 ± 1.5	11.4 ± 2.1	0.017	—	—
PS	2.5 ± 0.4	3.7 ± 0.3	3.3 ± 0.9	3.6 ± 0.9	—	0.017	—
PC	54.2 ± 0.8	52.7 ± 0.6	50.9 ± 2.7	51.0 ± 2.1	0.006	—	—
SM	2.0 ± 0.6	3.3 ± 0.7	3.1 ± 0.3	2.7 ± 0.6	—	—	—
Others	7.0 ± 0.9	6.5 ± 0.5	6.4 ± 0.7	6.4 ± 0.8	—	—	—
PC/PE ratio	2.1 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	2.1 ± 0.2	0.016	—	—

[a]Data are expressed as means ± SD of five mice. Abbreviations: See Table 1 and PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, total phospholipids; PS, phosphatidylserine; SM, sphingomyelin; Prot, protein effect.
[b]—, Not statistically significant.

TABLE 3

Effect of Dietary Protein and Cholesterol on Fatty Acid Composition (weight percent) of Liver Phosphatidylcholine in Mice[a]

	Dietary groups				Factorial effect		
Fatty acid	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
16:0	27.6 ± 1.8	25.6 ± 1.2	28.3 ± 1.0	27.4 ± 3.1	—[b]	—	—
16:1	1.9 ± 0.1	2.5 ± 0.1	1.7 ± 0.1	2.4 ± 0.4	—	0.001	—
18:0	11.5 ± 0.8	10.2 ± 0.8	11.9 ± 0.7	10.2 ± 0.7	—	0.001	—
18:1	10.1 ± 0.1	11.8 ± 1.0	9.6 ± 0.8	12.7 ± 2.6	—	0.003	—
18:2n-6	21.0 ± 1.1	26.4 ± 1.9	21.2 ± 1.1	26.0 ± 3.1	—	0.001	—
18:3n-6	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.004	—	0.028
20:3n-6	3.0 ± 0.4	4.1 ± 0.3	3.0 ± 0.3	3.8 ± 1.6	—	0.032	—
20:4n-6	10.2 ± 1.2	7.1 ± 0.7	10.2 ± 0.2	5.7 ± 0.4	—	0.001	—
18:3n-3	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.0 ± 0.3	—	0.004	—
20:5n-3	1.3 ± 0.3	1.3 ± 0.2	1.1 ± 0.1	1.2 ± 0.5	—	—	—
22:5n-3	0.5 ± 0.1	0.3 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.018	0.001	—
22:6n-3	10.2 ± 0.2	7.2 ± 0.4	10.0 ± 0.4	6.7 ± 1.7	—	0.001	—
Ratio							
Σ(n-6)/18:2	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	—	0.001	—
20:4/20:3	3.4 ± 0.4	1.7 ± 0.1	3.5 ± 0.4	1.8 ± 0.9	—	0.001	—

[a]Data are expressed as means ± SD of five mice. See Tables 1 and 2 for abbreviations and Σ(n-6) is the sum of 18:3n-6, 20:3n-6 and 20:4n-6.
[b]—, Not statistically significant.

effect on the distributin of fatty acids except for slight changes in the proportions of 18:3n-6 and 22:5n-3.

Table 4 shows the fatty acid composition of liver PE. CHOL feeding significantly affected the distribution of all fatty acids. The proportions of 18:1, 18:2n-6 and 20:3n-6 were increased, and those of 20:4n-6 and 22:6n-3 were reduced. Thus, CHOL feeding significantly depressed the ratio of (20:3n-6 + 20:4n-6)/18:2n-6 and 20:4n-6/20:3n-6. Dietary protein decreased the proportions of 16:1, whereas it increased those of 22:5n-3 and 22:6n-3. However, no dietary protein-dependent changes of the ratios of (20:3n-6 + 20:4n-6)/18:2n-6 and 20:4n-6/20:3n-6 were observed in liver PE.

Molecular species of liver PL. The distribution of molecular species in liver PC is shown in Table 5. Among the 18:2n-6 containing molecular species, the proportion of 16:0/18:2n-6, the major molecular species in liver PC, was significantly elevated in mice fed the CAS + CHOL as compared to that in those fed the CAS diet. The same trend was also observed between the SOY and the

TABLE 4

Effect of Dietary Protein and Cholesterol on Fatty Acid Composition (weight percent) of Liver Phosphatidylethanolamine in Mice[a]

Fatty acid	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
16:0	15.5 ± 1.1	13.7 ± 0.5	15.2 ± 0.2	14.4 ± 0.9	—[b]	0.002	—
16:1	0.8 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.8 ± 0.2	0.029	0.001	—
18:0	21.1 ± 0.8	19.4 ± 0.8	21.9 ± 0.2	19.0 ± 0.7	—	0.001	—
18:1	7.1 ± 0.9	9.6 ± 0.7	6.8 ± 0.3	9.5 ± 1.5	—	0.001	—
18:2n-6	6.3 ± 0.4	9.5 ± 0.9	5.9 ± 0.2	8.8 ± 0.5	—	0.001	—
18:3n-6	ND	ND	ND	ND	—	—	—
20:3n-6	1.0 ± 0.1	1.8 ± 0.1	1.0 ± 0.1	1.7 ± 0.5	—	0.001	—
20:4n-6	23.1 ± 1.1	22.2 ± 0.6	22.8 ± 0.4	21.9 ± 0.8	—	0.029	—
18:3n-3	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.0	0.8 ± 0.1	—	0.001	—
20:5n-3	1.8 ± 0.2	2.4 ± 0.2	1.8 ± 0.1	2.2 ± 0.6	—	0.005	—
22:5n-3	0.9 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	0.015	0.003	—
22:6n-3	19.9 ± 0.9	16.1 ± 0.5	20.5 ± 0.5	17.4 ± 1.3	0.031	0.001	—
Ratio							
Σ(n-6)/18:2	3.9 ± 0.4	2.6 ± 0.3	4.0 ± 0.2	2.7 ± 0.2	—	0.001	—
20:4/20:3	23.0 ± 0.8	12.2 ± 0.9	23.4 ± 0.8	13.9 ± 5.0	—	0.001	—

[a]Data are expressed as means ± SD of five mice. See Tables 1, 2 and 3 for abbreviations.
[b]—, Not statistically significant.

TABLE 5

Effect of Dietary Protein and Cholesterol on Liver Phosphatidylcholine Molecular Species (weight percent) in Mice[a]

Molecular species	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
16:0/16:1	0.1 ± 0.1	0.7 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.001	0.001	0.001
16:0/18:1	8.9 ± 0.5	8.9 ± 0.5	10.4 ± 0.5	11.4 ± 2.0	0.001	—[b]	—
16:1/18:1	0.2 ± 0.1	ND	ND	0.3 ± 0.1	—	—	0.001
16:0/18:2n-6	26.3 ± 2.7	34.1 ± 3.9	26.9 ± 2.2	30.3 ± 3.2	—	0.001	—
16:1/18:2n-6	1.3 ± 0.1	0.8 ± 0.0	1.1 ± 0.0	0.4 ± 0.1	0.001	0.001	—
18:0/18:2n-6	13.6 ± 0.7	14.1 ± 0.8	11.8 ± 0.6	12.0 ± 2.1	0.004	—	—
18:1/18:2n-6	2.1 ± 0.9	4.9 ± 0.8	1.5 ± 0.2	4.7 ± 1.6	—	0.001	—
18:2/18:2n-6	0.8 ± 0.0	1.8 ± 0.1	0.6 ± 0.0	2.1 ± 0.3	—	0.001	0.003
16:0/18:3n-6	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	—	—	—
16:0/20:3n-6	5.9 ± 0.6	6.7 ± 0.8	6.3 ± 0.5	11.3 ± 1.2	0.001	0.001	0.001
16:1/20:3n-6	ND	0.9 ± 0.0	ND	1.4 ± 0.2	0.001	0.001	0.001
18:0/20:3n-6	2.2 ± 0.1	2.7 ± 0.2	2.2 ± 0.1	3.1 ± 0.5	—	0.001	—
18:1/20:3n-6	0.1 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.5 ± 0.2	—	0.001	—
16:0/20:4n-6	11.3 ± 1.1	6.1 ± 0.8	11.4 ± 0.6	4.9 ± 1.2	—	0.001	—
18:0/20:4n-6	4.5 ± 1.5	1.9 ± 0.6	4.7 ± 0.7	1.5 ± 0.5	—	0.001	—
18:1/20:4n-6	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	—	—	—
16:0/22:4n-6	ND	0.2 ± 0.1	ND	ND	—	0.001	0.001
18:1/22:4n-6	ND	0.4 ± 0.1	ND	ND	0.001	0.001	0.001
16:0/20:5n-3	0.9 ± 0.3	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.3	—	—	—
16:0/22:5n-3	0.3 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.1	0.002	0.001	0.025
16:0/22:6n-3	16;0 ± 0.6	11.6 ± 0.4	16.1 ± 0.4	11.6 ± 1.8	—	0.001	—
18:0/22:6n-3	3.0 ± 0.6	1.0 ± 0.3	3.1 ± 0.6	1.6 ± 0.6	—	0.001	—
18:1/22:6n-3	0.9 ± 0.3	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.3	—	—	—

[a]Data are expressed as means ± SD of five mice. See Tables 1 and 2 for abbreviations.
[b]—, Not statistically significant.

SOY + CHOL groups. CHOL feeding also increased the proportions of 18:1/18:2n-6 and 18:2n-6/18:2n-6, but it did not affect the proportion of 18:0/18:2n-6. The latter was significantly higher in the CAS than in the SOY groups, independent of CHOL feeding.

The proportions of 20:3n-6 containing molecular species were generally higher in the CHOL-fed groups than in the CHOL-free groups. The difference was more pronounced in mice fed the SOY diet than the CAS diet. On the other hand, CHOL feeding decreased significantly the proportion of molecular species containing 20:4n-6 (16:0/20:4n-6 and 18:0/20:4n-6) and 22:6n-3 (16:0/22:6n-3 and 18:0/22:6n-3) irrespective of the source of dietary proteins.

In the liver PE fraction, the major PUFA were 20:4n-6 and 22:6n-3 (Table 4), and, as expected, the major molecular species contained 20:4n-6 or 22:6n-3 (Table 6). The relative proportions of the molecular species, 18:0/20:4n-6 and 18:1/20:4n-6 were not affected by either dietary protein or CHOL, whereas the proportions of molecular species, 16:0/20:4n-6, 16:0/22:6n-3 and 18:0/22:6n-3, were depressed significantly by CHOL feeding.

Among the 18:2n-6 containing molecular species, CHOL feeding increased the proportion of 18:0/18:2n-6 and 18:1/18:2n-6, but not 16:0/18:2n-6. The effect on the proportion of 18:0/18:2n-6 was more evident in mice fed the CAS diets than the SOY diet.

DISCUSSION

It has been reported previously that CAS in comparison with SOY enhanced Δ6 desaturase activity in rats (2–6). However, the results of the present study (Tables 3 and 4) as well as our previous report (27) have shown that dietary protein had no significant effect on fatty acid profiles in mice: the ratios of 20:4n-6/18:2n-6 in liver total PL were comparable between mice fed CAS and those fed SOY. These findings suggest that the response of Δ6 desaturase activity to dietary protein was less significant in mice than in rats. This may be due in part to a significantly lower level of Δ6 desaturase activity in mice than in rats (16).

It is also known that *in vivo* CHOL feeding suppressed the microsomal Δ6 desaturase activity in rat liver (28,29). Leikin and Brenner (30,31) have shown that desaturase activity in liver microsomes is affected by the ratios of CHOL/PL and PC/PE. Since *in vivo* CHOL feeding increased the CHOL/PL ratio in both rat (29) and mouse liver (Table 2), the inhibitory effect of CHOL feeding on linoleic acid metabolism in both rats and mice could be related to an increase in the CHOL/PL ratio (32). On the other hand, the inhibitory effect could not be related to a specific modulation of PC synthesis, since CHOL feeding increased the proportion of PC and the PC/PE ratio in rat liver (29) but not in mouse liver (Table 2). An alternative explanation for the inhibitory effect of dietary CHOL on Δ6 desaturase activity is that it might modify PC and PE fatty acid profiles in microsomal membranes, since the degree of unsaturation of PC and PE might modulate the enzyme activity in liver microsomes.

In rat liver PL, saturated fatty acids (16:0 and 18:0) are mainly located at the *sn*-1 position, and mono- and polyunsaturated fatty acids at the *sn*-2 position (33). In mice, the major saturated fatty acids in liver microsomal PC

TABLE 6

Effect of Dietary Protein and Cholesterol on Liver Phosphatidylethanolamine Molecular Species (weight percent) in Mice[a]

Molecular species	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
16:0/16:1	ND	0.4 ± 0.1	ND	0.3 ± 0.1	—[b]	0.001	—
16:0/18:1	0.1 ± 0.1	ND	0.1 ± 0.0	0.4 ± 0.1	0.001	0.001	0.001
16:1/18:1	ND	0.9 ± 0.2	0.8 ± 0.2	2.1 ± 0.4	0.001	0.001	—
16:0/18:2n-6	1.9 ± 1.2	2.0 ± 0.4	1.3 ± 0.3	1.4 ± 0.3	—	—	—
18:0/18:2n-6	1.9 ± 0.7	3.2 ± 0.4	2.0 ± 0.5	2.7 ± 0.3	—	0.001	—
18:1/18:2n-6	0.9 ± 0.3	2.8 ± 0.4	0.8 ± 0.1	2.5 ± 0.8	—	0.001	—
16:0/20:3n-6	0.4 ± 0.3	0.3 ± 0.1	0.2 ± 0.0	ND	0.001	0.044	—
18:0/20:3n-6	0.5 ± 0.2	1.4 ± 0.2	0.6 ± 0.1	1.1 ± 0.1	—	0.001	0.018
18:1/20:3n-6	ND	0.1 ± 0.0	ND	0.2 ± 0.0	—	0.001	—
16:0/20:4n-6	10.6 ± 1.8	8.7 ± 0.6	9.6 ± 0.4	9.0 ± 0.6	—	0.020	—
16:1/20:4n-6	ND	0.2 ± 0.1	ND	0.2 ± 0.1	—	0.001	—
18:0/20:4n-6	32.5 ± 5.2	36.5 ± 1.8	34.4 ± 2.0	34.1 ± 1.5	—	—	—
18:1/20:4n-6	5.1 ± 1.3	5.9 ± 0.8	5.1 ± 0.3	5.6 ± 0.5	—	—	—
18:2/20:4n-6	ND	0.1 ± 0.0	ND	0.2 ± 0.1	—	0.001	—
16:0/22:5n-6	0.1 ± 0.0	ND	0.1 ± 0.0	ND	0.002	0.001	0.001
16:0/18:3n-3	ND	ND	0.2 ± 0.0	ND	0.001	0.001	0.001
18:0/18:3n-3	0.6 ± 0.4	ND	ND	ND	0.004	0.004	0.004
16:0/20:5n-3	0.5 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	1.0 ± 0.2	—	0.001	—
18:0/20:5n-3	1.0 ± 0.2	1.4 ± 0.1	1.0 ± 0.1	1.2 ± 0.4	—	0.017	—
18:1/20:5n-3	ND	0.4 ± 0.1	ND	0.4 ± 0.2	—	0.001	—
16:0/22:5n-3	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	—	0.011	—
16:0/22:6n-3	27.0 ± 0.9	22.9 ± 0.8	24.7 ± 1.3	23.4 ± 0.8	—	0.001	0.005
18:0/22:6n-3	9.2 ± 1.2	5.9 ± 0.4	10.6 ± 0.7	6.8 ± 1.2	0.021	0.001	—
18:1/22:6n-3	6.2 ± 1.8	5.1 ± 0.3	6.1 ± 0.6	5.6 ± 1.0	—	—	—

[a]Data are expressed as means ± SD of five mice. See Tables 1 and 2 for abbreviations.
[b]—, Not statistically significant.

and PE were also 16:0 and 18:0 (Tables 3 and 4). The major PUFA in PC were C_{18} and C_{20}, and in PE were C_{20} and C_{22} (Tables 5 and 6). To compare the level of incorporation of PUFA into 16:0 and 18:0 containing molecular species in PC and PE, we have calculated the relative distribution of PUFA in 16:0 and 18:0 containing molecular species. Table 7 shows that the ratio of 16:0-containing to 18:0-containing molecular species in liver PC ranged from 2 to 5. This finding indicated that molecular species in liver microsomes containing either type of PUFA were approximately two to five times more likely to also contain 16:0 than 18:0. In the PE fraction, the ratio of 16:0-containing to 18:0-containing molecular species ranged from 0.3 to 1.0 when 18:2n-6, 20:3n-6, 20:4n-6 or 20:5n-3 was also present in the same molecule (Table 7). This result suggests that C_{18} and C_{20} PUFA were incorporated preferentially into PE molecular species containing 18:0 rather than 16:0. However, not all PUFA behave the same way. The ratios of 16:0-containing to 18:0-containing molecular species in PC and PE ranged from 2 to 5 when 22:6n-3 was also present in the same molecule, suggesting that C_{22} PUFA was incorporated preferentially into PC and PE molecular species containing 16:0 rather than those containing 18:0. These observations are also in consistence with the report that the total carbon number of fatty acids in different PE molecular species is around 38, and that in PC molecular species is 36 to 38 (34).

CHOL feeding increased the ratio of 16:0-containing to 18:0-containing molecular species in liver PC, particularly in those containing 20:4n-6. Since CHOL feeding lowered the proportions of 20:4n-6 in both 16:0- and 18:0-containing molecular species, the increased ratio of 16:0-containing to 18:0-containing molecular species was attributed to a greater decrease of 18:0-containing molecular species. Similarly, CHOL feeding suppressed the proportion of 22:6n-3-containing molecular species in both PC and PE (Tables 5 and 6). CHOL feeding also reduced the proportion of 18:0/22:6n-3 molecular species to a greater extent than that of 16:0/22:6n-3 (Table 7).

In order to examine whether there exists any specific pattern in the distribution of common molecular species between PC and PE, and whether this pattern was modulated by dietary protein or CHOL feeding, we have also calculated the relative distribution of common molecular species in both PC and PE fractions. Table 8 shows that PC, as compared to PE, contained a greater proportion of molecular species containing 18:1, 18:2n-6 and 20:3n-6. The molecular species, such as 16:0/20:4n-6 and 16:0/20:5n-3, were evenly distributed between PC and PE, whereas 18:0/20:4n-6 and molecular species containing 22:6n-3 constituted a greater proportion in PE than in PC. Dietary protein did not affect the relative distribution of common molecular species in PC and PE. However, CHOL feeding decreased the relative distributions of molecular species containing 20:4n-6, 20:5n-3 and 22:6n-3, and those of 18:0/18:2n-6 in PC and PE. The reduction was due to either an increase in the proportions of the PE fraction, such as 18:0/18:2n-6 and 16:0/20:5n-3, or a decrease in the PC fraction, such as 16:0/20:4n-6. Thus, CHOL feeding affects differently the incorporation of long-chain PUFA into PL classes (PC and PE).

In summary, the results of the present study on mouse liver show that CHOL feeding inhibited linoleic acid metabolism without affecting the concentration of PC, whereas animal protein raised the relative concentration of PC in total PL, but exerted little or no effect on linoleic acid metabolism. CHOL feeding, and to a lesser extent animal protein, affected the constituent molecular species of the PC and PE fractions that were characteristically different. The CHOL-induced effect is probably expressed through modulation of the incorporation of long-chain PUFA into PC and PE which, in turn, changes the physical properties of the microsomal membrane.

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TABLE 7

Effect of Dietary Protein and Cholesterol on Ratio of Molecular Species of Liver Phosphatidylcholine and of Liver Phosphatidylethanolamine in Mice[a]

Molecular species compared	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
	(ratios)						
Phosphatidylcholine							
16:0/18:2n-6 *vs.* 18:0/18:2n-6	1.9 ± 0.2	2.4 ± 0.4	2.3 ± 0.3	2.6 ± 0.7	—[b]	—	—
16:0/20:3n-6 *vs.* 18:0/20:3n-6	2.7 ± 0.2	2.5 ± 0.4	2.8 ± 0.3	3.8 ± 1.0	0.020	—	—
16:0/20:4n-6 *vs.* 18:0/20:4n-6	2.9 ± 0.9	3.4 ± 0.8	2.5 ± 0.5	3.4 ± 0.2	—	0.034	—
16:0/22:6n-3 *vs.* 18:0/22:6n-3	5.4 ± 1.1	12.0 ± 2.5	5.3 ± 0.8	8.4 ± 3.9	—	0.001	—
Phosphatidylethanolamine							
16:0/18:2n-6 *vs.* 18:0/18:2n-6	1.0 ± 0.7	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.2	—	—	—
16:0/20:3n-6 *vs.* 18:0/20:3n-6	1.0 ± 0.7	0.2 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.013	0.003	—
16:0/20:4n-6 *vs.* 18:0/20:4n-6	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	—	0.047	—
16:0/20:5n-3 *vs.* 18:0/20:5n-3	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.006	0.001	—
16:0/22:6n-3 *vs.* 18:0/22:6n-3	3.0 ± 0.5	3.9 ± 0.3	2.3 ± 0.1	3.5 ± 0.6	0.030	0.001	—

[a]Data are expressed as means ± SD of five mice. See Tables 1 and 2 for abbreviations.
[b]—, Not statistically significant.

TABLE 8

Relative Distribution of Specific Molecular Species in Phosphatidylcholine as Compared to Phosphatidylethanolamine in Mouse Liver[a]

Molecular species compared (PC *vs.* PE)	Dietary groups				Factorial effect		
	CAS	CAS + CHOL	SOY	SOY + CHOL	Protein	CHOL	Prot/CHOL
	(ratios)						
16:0/18:1	75.6 ± 29.6	INF	84.8 ± 21.5	28.9± 4.0	—[c]	—	—
16:0/18:2n-6	20.2 ± 14.7	17.8 ± 2.5	20.6 ± 3.8	22.0 ± 3.1	—	—	—
18:0/18:2n-6	8.1 ± 3.2	4.5 ± 0.8	6.1 ± 1.6	4.5 ± 0.6	—	0.009	—
18:1/18:2n-6	2.5 ± 1.0	1.8 ± 0.5	2.0 ± 0.4	1.9 ± 0.4	—	—	—
16:0/20:3n-6	20.4 ± 15.0	21.6 ± 3.0	36.0 ± 6.7	INF[b]			
18:0/20:3n-6	5.5 ± 2.2	2.0 ± 0.3	4.2 ± 1.1	2.7 ± 0.4	—	0.001	—
16:0/20:4n-6	1.1 ± 0.3	0.7 ± 0.1	1.2 ± 0.1	0.5 ± 0.1	—	0.001	—
18:0/20:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	—	0.001	—
18:1/20:4n-6	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	—	0.039	—
16:0/20:5n-3	1.9 ± 0.6	0.7 ± 0.2	1.4 ± 0.4	0.7 ± 0.2	—	0.001	—
16:0/22:6n-3	0.6 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.5 ± 0.1	—	0.001	—
18:0/22:6n-3	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	—	0.003	—

[a]Data are expressed as means ± SD of five mice. See Tables 1 and 2 for abbreviations.
[b]INF: This molecular species was not detected in the phosphatidylethanolamine fraction. No factorial analysis was carried out.
[c]—, Not statistically significant.

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Effect of Dietary Fish Oil on Blood Levels of Free Fatty Acids, Ketone Bodies and Triacylglycerol in Humans

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Although the reduction of serum triacylglycerol concentrations by dietary fish oil is a well-known effect, the exact mechanism of this effect has not been previously studied in human subjects. Therefore, the aim of this study was (i) to examine the effect of short-term fish oil supplementation on blood concentrations of ketone bodies, free fatty acids and triacylglycerol in healthy humans and (ii) to verify whether the observed relationships between these variables would be consistent with reduced lipolysis and/or enhanced hepatic fatty acid oxidation after fish oil supplementation. Twenty subjects (21–23 years, normal liver function tests) were randomly divided into two groups to supplement their usual diet with either 30 g/d of fish oil (n = 11) or olive oil (n = 9). Venous blood samples were drawn after an overnight fast, before and after 1, 3 and 7 d of fish oil/olive oil supplementation. Blood concentrations of triacylglycerol and free fatty acids decreased consistently after fish oil supplementation; the reduction was already significant after one day of fish oil ($P < 0.001$ for triacylglycerol and $P = 0.01$ for free fatty acids). In contrast, neither of these blood values changed after olive oil supplementation ($P > 0.10$). No significant changes in glucose, insulin or ketone body levels were observed in either group after supplementation. After fish oil, but not after olive oil supplementation, the ratio of blood ketone body levels to free fatty acid levels increased significantly ($P < 0.05$). Furthermore, after fish oil supplementation only, free fatty acid levels were significantly correlated with levels of ketone bodies (day 7 of supplementation: $r = 0.90$, $P < 0.001$) and triacylglycerol (maximum value on day 3: $r = 0.77$, $P < 0.01$). These findings suggest that reduced lipolysis and increased hepatic β-oxidation/ketogenesis may contribute to reduced triacylglycerol levels after ω3 fatty acid supplementation in humans. Turnover studies are needed in order to further quantitate these processes.

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The ω3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in fish oil have aroused increasing interest over the past two decades because of their potential role in the prevention and treatment of cardiovascular disease (1,2). One of the well-described effects of ω3 fatty acids from fish oil on humans is the reduction of serum triacylglycerol concentrations (3–7). The exact mechanism of this effect on humans is unknown. Animal studies have mainly focused upon the inhibitive effects of ω3 fatty acids on very low density lipoprotein (VLDL) formation and secretion (8,9), especially triacylglycerol synthesis (10–15). Recently, it was suggested that a slower formation of plasma VLDL triacylglycerols in rats fed fish oil may be due to a faster rate of hepatic fatty acid oxidation (16). There are also recent animal data suggesting that ω3 fatty acids may reduce lipolysis from adipose tissue, thus limiting the availability of free fatty acids as a substrate for hepatic triacylglycerol formation (17,18).

Although the ultimate goal of animal studies is to gain insight into potential mechanisms of the action of ω3 fatty acids in humans, no human studies on the effect of ω3 fatty acids on lipolysis and ketone body production have been reported so far. Therefore, the aim of the present study was to investigate the effects of dietary fish oil on blood levels of free fatty acids, ketone bodies and triacylglycerol in healthy human subjects, and to verify whether the observed relationships between these variables would be consistent with reduced lipolysis and/or enhanced hepatic fatty acid oxidation after fish oil supplementation.

MATERIALS AND METHODS

Subjects and study design. The study protocol was approved by the Research Ethics Committee of the University Hospital of Rotterdam, and written informed consent was obtained from all subjects prior to the study. Eligible for the study were healthy medical students aged 21–23 yr who were on normal, nonslimming diets with, at most, one meal of fish per week. All subjects had normal liver function tests and took no fish from one week prior to the study and throughout. Twenty-two subjects were randomly distributed over an experimental and a placebo group in a double-blinded fashion. Two subjects from the placebo group did not show up on the first day of the study, leaving eleven subjects in the experimental group and nine in the placebo group. Subjects in the experimental group took 30 g of MaxEPA fish oil per day, containing a total of 9 g ω3 fatty acids per day (EPA, 5.4 g/d; DHA, 3.6 g/d) for 7 d. Subjects in the placebo group took 30 g of olive oil per day during the same period. Capsules of fish oil and olive oil (indistinguishable by addition of peppermint oil and ferrous oxide coloring) were kindly provided by Seven Seas Ltd. (Hull, United Kingdom), and taken as 3 × 10 capsules per day with the main meals.

Measurements. Following an overnight fast of approximately 12 h, subjects came to the laboratory between 8 and 9 a.m., before (day 0) and after supplementation (days 1, 3 and 7). On these days, subjects were weighed, and venous blood samples were drawn from the forearm. Height was measured once preceding the study.

Laboratory analyses. Glucose concentrations in whole blood were determined by an enzymatic assay using an Epos 5060 analyzer (Merck-Eppendorf, Darmstadt, Germany) with reagents from Boehringer (Mannheim, Germany). Triacylglycerol in serum was determined enzymatically on a Chem-1 analyzer (Bayer-Technicon,

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Abbreviations: CPT, carnitine palmitoyl transferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; VLDL, very low density lipoprotein(s).

Tarrytown, New York). Free fatty acids were determined in ethylenediaminetetraacetic acid-treated plasma using an enzymatic colorimetric method (NEFA C test kit; Wako Chemicals, Neuss, Germany). A conversion factor of 0.282 was used (derived from the molecular weight of oleic acid) for conversion of these values (from μmol/L into mg/L). For determination of ketone bodies in whole blood, 4 mL of blood was poured immediately into a glass bottle containing 4 mL ice-cold perchloric acid, and, after neutralization with KOH (19), stored at −20°C until the study was completed. Acetoacetate and β-hydroxybutyrate concentrations were determined according to Mellanby and Williamson (19) and Williamson and Mellanby (20), respectively. Insulin in serum was determined using a commercial radioimmunoassay test kit (Medgenix, Brussels, Belgium).

Compliance. For each subject, the number of capsules taken per day was calculated by pre- and post-weighing supplied capsules, and used as an indicator of compliance.

Statistical analysis. Blood values are expressed as means ± SEM, except for free fatty acids, ketone bodies and the ketone body/free fatty acid ratio. As the latter showed a skewed distribution which best fitted normal distribution after log-transformation, log-transformed data were used for statistical calculations of these parameters, and data for these parameters are presented as geometrical means and range. Changes in blood values within one group were tested for statistical significance using Student's paired *t*-test. Comparison of the effects of fish oil *vs.* olive oil supplementation on blood values was tested after adjustment for baseline values by analysis of covariance. Mean values and 1, 3 and 7 d of supplementation were used for the previously mentioned statistical calculations. For comparing residual variances between groups, the standard F-test was used (21). Changes in residual standard deviation after supplementation within one group were tested by 11 "repeated measurements analysis of variance" using module 5V of BMDP (22). *P* values less than 0.05 were considered statistically significant.

RESULTS

Anthropometric measurements. Baseline values of body weight and height were similar in the fish oil group and the olive oil group. The quetelet index was 21.7 ± 0.6 kg/cm² (mean ± SEM) in the fish oil group and 22.5 ± 1.3 kg/cm² in the olive oil group. No relation between quetelet index and baseline values of any of the measured blood parameters was detected. No significant changes in weight were observed in either group during the study (fish oil group, 0.2 ± 0.1 kg; olive oil group, −0.5 ± 0.3 kg).

Compliance. Participants had been instructed to take 30 g of the provided supplement per day. The estimated "actual" intake ranged from 29–31 g/d in the fish oil group and from 29–32 g in the olive oil group, indicating good compliance.

Blood values. Blood glucose and insulin concentrations did not change systematically after fish or olive oil supplementation (Table 1). Triacylglycerol concentrations decreased by 24% ($P < 0.001$) within 1 d of fish oil supplementation. After 7 d of fish oil supplementation, triacylglycerol had decreased by 46% ($P < 0.001$), as compared to baseline (Table 1). Olive oil supplementation had no significant effect on triacylglycerol levels ($P > 0.10$; Table 1). The effects of fish oil and olive oil supplementation on triacylglycerol levels were significantly different ($P = 0.001$).

Free fatty acid concentrations decreased by 39% ($P = 0.01$) within 1 d of fish oil supplementation and remained significantly depressed at 3 and 7 d of supplementation for each ($P < 0.05$ as compared to baseline; Table 2). In contrast, no significant change in free fatty acid levels was observed after olive oil supplementation ($P > 0.10$). The effects of fish oil and olive oil supplementation on free fatty acid levels were significantly different ($P = 0.03$). No significant changes in ketone body levels were observed after either fish oil or olive oil supplementation ($P > 0.10$; Table 2).

TABLE 1

Blood Values of Glucose, Insulin and Triacylglycerol in Healthy Human Subjects Before and After Dietary Supplementation with Fish Oil or Olive Oil[a]

	Before supplementation	After supplementation for			*P* value	
		1 d	3 d	7 d	Change within groups[b]	Change compared between groups[c]
Glucose (mmol/L)						
Fish oil group	4.2 ± 0.1	4.3 ± 0.1	4.3 ± 0.1	4.0 ± 0.1	NS[d]	NS
Olive oil group	4.4 ± 0.2	4.6 ± 0.2	4.3 ± 0.1	4.3 ± 0.1	NS	
Insulin (pmol/L)						
Fish oil group	102 ± 9	104 ± 11	84 ± 10	97 ± 13	NS	NS
Olive oil group	121 ± 12	97 ± 10	92 ± 9	99 ± 9	NS	
Triacylglycerol (mmol/L)						
Fish oil group	0.96 ± 0.08	0.73 ± 0.06	0.64 ± 0.05	0.61 ± 0.06	< 0.001	0.001
Olive oil group	1.04 ± 0.17	0.98 ± 0.13	1.04 ± 0.10	1.07 ± 0.14	NS	

[a]Healthy human subjects were randomized to take a daily supplement of either 30 g fish oil (9 g ω3 fatty acids; n = 11) or 30 g olive oil (n = 9) for 7 d. Each figure represents the arithmetic mean ± SEM.
[b]Paired *t*-test (mean value of 1, 3 and 7 d of supplementation *vs.* baseline value).
[c]Effects of fish oil *vs.* olive oil supplementation were tested using mean values of 1, 3 and 7 d of supplementation, adjusting for baseline values by analysis of covariance.
[d]NS, not significant.

TABLE 2

Blood Values of Free Fatty Acids and Ketone Bodies in Healthy Human Subjects Before and After Dietary Supplementation with Fish Oil or Olive Oil[a]

	Before supplementation GM (range)	After supplementation for 1 d GM (range)	3 d GM (range)	7 d GM (range)	*P* value: Change within groups[b]	*P* value: Change compared between groups[c]
Free fatty acids (mg/L)						
Fish oil group	92 (54-141)	56 (35-91)	57 (25-128)	58 (28-195)	0.02	0.03
Olive oil group	99 (37-236)	84 (18-183)	89 (50-160)	93 (45-158)	NS[d]	
Ketone bodies (μmol/L)						
Fish oil group	46 (27-137)	38 (24-61)	39 (23-58)	42 (22-135)	NS	NS
Olive oil group	73 (31-267)	56 (31-111)	58 (30-247)	57 (38-97)	NS	

[a]Study design: see footnote a to Table 1. Each figure represents the geometrical mean (GM) and range.
[b]Paired *t*-test (mean value of 1, 3 and 7 d of supplementation *vs.* baseline value).
[c]Effects of fish oil *vs.* olive oil supplementation were tested using mean values of 1, 3 and 7 d of supplementation, adjusting for baseline values by analysis of covariance.
[d]NS, not significant.

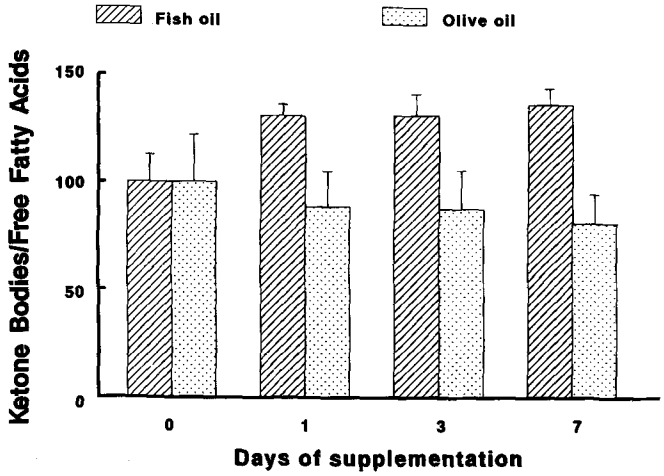

FIG. 1. Ratios of blood ketone body concentrations to plasma free fatty acids before and after 1, 3 and 7 d of fish oil or olive oil supplementation. Healthy human subjects took a daily supplement of either 30 g fish oil (9 g ω3 fatty acids; n = 11) or 30 g olive oil (n = 9). Boxes and bars represent means and SEM, respectively, of logarithmic values (expressed as a percentage of values before supplementation). Significance of change after fish oil supplementation (paired *t*-test of mean value of 1, 3 and 7 d of supplementation *vs.* baseline value), $P < 0.05$; olive oil supplementation, $P > 0.10$. Comparison of the effects of fish oil *vs.* olive oil supplementation (mean values of 1, 3 and 7 d of supplementation, adjusting for baseline values by analysis of covariance), $P = 0.02$.

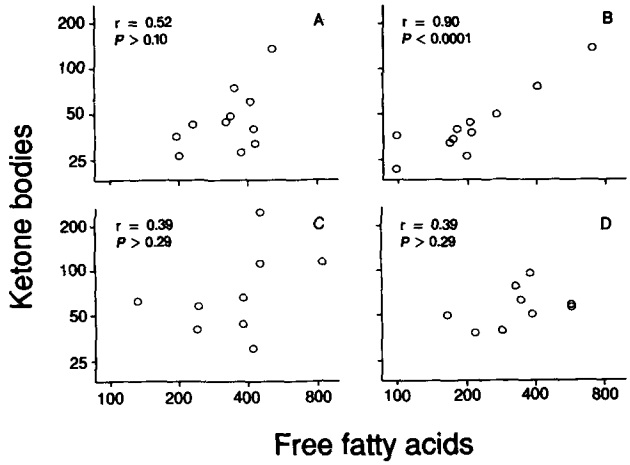

FIG. 2. Blood ketone body concentrations (μmol/L) as a function of plasma free fatty acid concentrations (μmol/L) before and after supplementation with fish or olive oil; (A) before fish oil supplementation, (B) after 7 d of fish oil supplementation, (C) before olive oil supplementation, (D) after 7 d of olive oil supplementation. The methods used are those given in the caption for Figure 1.

Ratios of ketone body levels relative to free fatty acid levels (Fig. 1) increased significantly after fish oil supplementation ($P < 0.05$), but did not change after olive oil supplementation ($P > 0.10$). Again, the effects of fish oil and olive oil supplementation were significantly different ($P = 0.02$).

In Figure 2, ketone bodies are plotted as a function of free fatty acids before and after seven days of supplementation with fish oil or olive oil. No significant correlation between free fatty acid levels and ketone body concentrations was observed in either group before supplementation, or after olive oil supplementation ($P > 0.10$). In contrast, these two parameters were highly correlated after seven days of fish oil supplementation ($r = 0.90$, $P < 0.001$). The residual standard deviation of blood ketone body levels against free fatty acid levels was consistently reduced after seven days of fish oil supplementation ($P \leq 0.01$), and residual standard deviation was significantly lower after fish oil supplementation than after olive oil supplementation (day 7: $P < 0.001$). As the total variation in ketone body levels did not increase after fish oil supplementation (Table 2 and Fig. 2), the increase in correlation between free fatty acid and ketone body levels after fish oil supplementation (and the difference in correlation when compared with olive oil supplementation) was exclusively attributed to a reduction in residual standard deviation after fish oil supplementation.

Free fatty acid levels were not significantly correlated with triacylglycerol concentrations in either group before supplementation or after olive oil supplementation ($P > 0.10$). An increased correlation was observed after seven days of fish oil supplementation which, however, did not reach statistical significance ($r = 0.52$; $P = 0.10$). After three days of fish oil supplementation, the correlation between free fatty acid and triacylglycerol levels was highly significant ($r = 0.77$, $P < 0.01$).

DISCUSSION

Although it is well known that marine ω3 fatty acids reduce serum triacylglycerol concentrations in humans (3-7), the mechanism underlying this effect is not completely understood. Most of the effects described so far, observed in animal models *in vivo* and *in vitro*, were related to reduced production and secretion of triacylglycerol-rich lipoproteins (VLDL) by the liver. Effects of ω3 fatty acids on nearly every major pathway of hepatic fatty acid metabolism have been demonstrated. Reduction of triacylglycerol synthesis by ω3 fatty acids has been attributed to reduced activities of phosphatidate phosphohydrolase (10,11) and acyl-coenzyme A:1,2-diacylglycerol acyltransferase (12,23), and to a diversion toward phospholipid synthesis (13-15,24). At high dosage of ω3 acids, an inhibition of VLDL assembly and/or secretion has been reported (9).

A number of animal studies have also demonstrated increased hepatic β-oxidation capacity and ketone body production after fish oil feeding (11,24-30). Surette *et al.* (31) observed an increased activity of hepatic carnitine palmitoyltransferase (CPT) in fish oil-fed Syrian hamsters, which was highly correlated ($r = -0.97$) with the diet-induced change in serum triacylglycerol levels. Wong *et al.* (25) observed a decreased sensitivity of mitochondrial CPT to inhibition by malonyl-CoA.

Recently, attention has been drawn to the possibility that fish oil decreases peripheral lipolysis from adipose tissue, thereby reducing the availability of substrate for triacylglycerol synthesis in the liver (17,18,27). The activities of lipoprotein lipase and hepatic lipase, enzymes which are involved in VLDL catabolism, are unaffected by fish oil supplementation (6).

Because there is little information about these processes in humans, a randomized, placebo-controlled study on the effects of fish oil supplementation on blood values of free fatty acids, ketone bodies and triacylglycerol, and the relationship between these blood values was carried out. Results demonstrate that fish oil supplementation induced a rapid parallel reduction in blood concentrations of triacylglycerol and free fatty acids, whereas ketone body levels remained constant. Changing blood values were not due to increased fat consumption *per se*, because supplementation with placebo (olive oil) did not affect either free fatty acid or triacylglycerol levels.

Although the reduction in free fatty acid levels after fish oil supplementation has not been systematically studied in healthy human subjects, Singer *et al.* (17) recently reported that dietary supplementation with ω3 fatty acids resulted in a marked decrease in free fatty acid levels during a standard glucose tolerance test in hyperlipidemic patients. Otto *et al.* (18) observed a dose-dependent reduction in plasma free fatty acids by ω3 fatty acids in rats fed *ad libitum*, but not in rats fasted overnight. Since insulin concentration were constant and glucose concentrations increased, the authors (18) concluded that lower plasma free fatty acid levels could not be explained by either increased insulin concentrations or improved insulin sensitivity of adipose tissue. Preliminary data from another recent study (32) in rats fed marine ω3 fatty acids confirmed a marked reduction in free fatty acid levels. In addition, a significant reduction of fatty acid mobilization from epididymal adipocytes of these rats was demonstrated (32). Our finding of reduced blood values of free fatty acids in spite of constant glucose and insulin levels after fish oil supplementation is consistent with these recent reports.

In their studies with rats, Otto *et al.* (18) observed a positive correlation ($r = 0.71$) between blood values of free fatty acids and triacylglycerol. A similar correlation ($r = 0.77$) was observed in the present study after three days of fish oil supplementation. This would suggest that the lowering of plasma free fatty acids by dietary fish oil may have contributed to the decrease in serum triacylglycerol levels.

In spite of the decrease in plasma free fatty acid levels after fish oil supplementation, we did not detect any significant changes in blood ketone body levels. As a consequence, the ratio of ketone bodies to free fatty acids increased significantly after fish oil supplementation. It is unlikely that this was caused by decreasing ketone body removal from the blood, as ketone body uptake by peripheral tissues is thought to be controlled by the ketone body concentrations presented to them (33,34). Arterial ketone body levels closely reflect hepatic ketone body production (35). Therefore, the increased ratio of ketone bodies to free fatty acids, combined with the markedly high correlation ($r = 0.90$) between these blood values after fish oil supplementation, suggests an increased capacity for fatty acid oxidation and/or ketogenesis in the liver. This suggestion is consistent with animal studies that demonstrated increased β-oxidation and ketogenesis by dietary ω3 fatty acids (11,24-31).

If increased hepatic β-oxidation and ketogenesis would not be counteracted by reduced lipolysis from adipose tissue, ketosis would occur (36). It is speculated that the combination of decreased lipolysis and increased capacity or hepatic β-oxidation/ketogenesis may be a concerted mechanism whereby fish oil reduces triacylglycerol formation in the liver without, however, simultaneously inducing ketosis.

In conclusion, our study shows that dietary fish oil, in addition to its well-known effect of reducing serum triacylglycerol concentrations, induces a rapid reduction in blood free fatty acids, and a parallel rise in the ratio of ketone bodies relative to free fatty acids. These effects appear not to be due to changes in insulin concentration or sensitivity, and because they occur within one day of supplementation, they are unlikely to be related to altered membrane composition by ω3 fatty acids. Our findings are consistent with the notion that reduced lipolysis and increased hepatic β-oxidation/ketogenesis may contribute to reduced triacylglycerol levels after ω3 fatty acid supplementation in humans. Turnover studies are needed in order to provide further quantitation of these processes.

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Lipid Peroxidation and Antioxidant Status Is Affected by Different Vitamin E Levels When Feeding Fish Oil

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The protective role of vitamin E and changes in the status of several physiological antioxidants after feeding rats a fish oil diet were investigated. Six-week-old male Sprague-Dawley rats were divided into four groups and fed experimental diets for 8 wk. Three fish oil (FO) groups were fed a menhaden fish oil and soybean oil (SO) (9:1) mixture as 10% (w/w) of the diet. These groups were provided with ≤3, 45 or 209 IU of vitamin E/kg diet. One SO group was used as control and was fed ≤45 IU of vitamin E/kg diet. Plasma vitamin E levels, when expressed as vitamin E per mL plasma, were extremely low in the group fed FO and ≤3 IU of vitamin E, and were lower in the groups fed FO than in the group fed SO. However, plasma vitamin E levels when expressed per mg plasma lipid were higher in the FO groups provided with ≤45 and 209 IU of vitamin E than in the SO group. Compared with the SO group, plasma levels of thiobarbituric acid reactive substances (TBARS), when expressed per mg lipid, were higher in the three FO groups, plasma retinol levels were lower in the FO groups provided with ≤3 and 45 IU of vitamin E, and ascorbic acid levels were lower only in the FO group provided with ≤3 IU of vitamin E. Blood glutathione (GSH) levels were lower in all three FO groups than in the SO group. Liver vitamin E levels increased as the dietary level of vitamin E increased, but all FO groups had higher liver levels of TBARS than the SO group. The dietary vitamin E levels were correlated positively with plasma vitamin E ($r = 0.71$) and negatively with TBARS in both the plasma and liver of rats fed FO. Among the antioxidants measured, correlations were found between plasma retinol and vitamin C ($r = 0.64$), and plasma vitamin C, uric acid ($r = 0.72$) and blood GSH ($r = 0.60$). Weaker correlations were found between plasma retinol, uric acid and blood GSH. It is concluded that vitamin E requirements are higher when feeding fish oil. Vitamin E seems necessary to prevent enhanced lipid peroxidation and to maintain appropriate levels of other physiological antioxidants.

Lipids 29, 47-52 (1994).

During the past two decades, epidemiologic studies on animals and humans have shown the beneficial effects of marine oils in preventing or retarding coronary heart disease and other chronic illnesses (1). Observed positive effects include the lowering of blood lipid levels, especially of triglyceride, the lowering of blood pressure, and reduced platelet aggregation and inflammation (1). However, increased levels of long-chain n-3 fatty acids may render membranes more susceptible to oxidation and may increase the requirement for antioxidants. Lipid peroxidation , *in vivo* (2-6) and *in vitro* (7,8), has been shown to increase in animals fed fish oil (FO) as well as in isolated cells enriched with eicosapentaenoic acid and docosahexaenoic acid (9); tissue vitamin E levels concomitantly decreased (2-4). It also has been shown in human studies that plasma vitamin E levels were decreased upon long-term FO supplementation (10), and that this effect was alleviated by higher dietary vitamin E intake (11). These studies, however, did not lead to recommendations on appropriate vitamin E supplementations in order to maintain normal antioxidant status during fish oil feeding.

Although vitamin E is the most important cellular antioxidant (12), ascorbic acid (13,14), retinol (15), carotenoid (16), glutathione (14,16) and uric acid (14) are also important antioxidants that work cooperatively with vitamin E. Following consumption of vitamin E, changes in the status of these antioxidants do occur, which may have detrimental effects of peroxidation and may impair normal functions.

In the present study we investigated the effects of FO and of vitamin E supplementation on lipid peroxidation as well as the relationship between various physiological antioxidants in plasma and liver of rats fed FO.

MATERIALS AND METHODS

Diets and animals. Starch and glucose were purchased from Je-il Feed Co. (Seoul, Korea). Vitamin-free casein, mineral mix, cellulose, choline, inositol and DL-methionine were purchased from Teklad Test Diets (Madison, WI); DL-α-tocopheryl acetate was obtained from Sigma Chemical Co. (St. Louis, MO). Menhaden oil (MO) was kindly donated by Zapata Haynie Inc. (Reedville, VA), and soybean oil (SO) was purchased from a local market. Tertiary butylhydroxyquinone (TBHQ, 0.02%; Eastman Kodak, Rochester, NY) was added to keep MO and SO from deteriorating. Other precautions for maintaining the freshness of the diets were followed (17). To prevent linoleic acid deficiency in feeding MO, one part SO was mixed with nine parts MO for the FO diet at the level of 10% (w/w) fat in the diet. The composition of the diets is shown in Table 1. Table 2 shows the fatty acid compositions and peroxide values of the two dietary oils. Fatty acid composition was analyzed by gas chromatography as described below, and peroxide values were determined according to AOAC methods 28.025 and 28.026 (18). In the FO diet, the level of linoleic acid was 1.07% (w/w) and comprised about 2.2 cal%. Since SO and MO contained ≤26 IU vitamin E and ≤0.8 IU vitamin E per 100 g oil, respectively, the amounts of DL-α-tocopherol were adjusted to the three different levels (≤3, 45, 209 IU per kg diet) of vitamin E in the FO diets and ≤45 IU vitamin E per kg of the SO diet. Six-week-old male Sprague-Dawley rats

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Abbreviations: BHT, butylated hydroxytoluene; FO, fish oil; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; MO, menhaden oil; PUFA, polyunsaturated fatty acid; SO, soybean oil; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroxyquinone; UV, ultraviolet.

TABLE 1

Compositions of Experimental Diets[a]

	Vitamin E (IU/kg)			
	≤45	3	45	209
Components	SO diet	FO (MO/SO = 9:1) diets		
	per kg			
Starch	370 g			
Glucose	190 g			
Casein	240 g			
Mineral mix (AIN-76A)	40 g			
Vitamin E-free vitamin mix[b]	10 g			
Cellulose	40 g			
Choline	0.5 g			
Inositol	0.5 g			
DL-Methionine	2.2 g			
Soybean oil	100	10	10	10 g
Menhaden oil		90	90	90 g
DL-α-Tocopheryl acetate	19	0	42	206 mg

[a]Four experimental diets were formulated using menhaden oil (MO) and soybean oil (SO) as fat sources and three different levels of vitamin E. SO was used as the sole fat source for the SO diet while an MO and SO (9:1) mixture was used in three FO diets at the level of 10% (w/w) to provide 2.2 cal% as linoleic acid. Since SO and MO contained ≤26 and ≤0.8 IU vitamin E per 100 g oil, respectively, the amounts of DL-α-tocopherol were adjusted to the three different levels (≤3, 45, 209 IU per kg diet) of vitamin E in the FO diets, and to ≤45 IU vitamin E per kg in the SO diet.

[b]Vitamin E-free vitamin mix (per kg) contained thiamin•HCl, 0.6 g; riboflavin, 0.6 g; pyridoxin•HCl, 0.7 g; nicotinic acid, 3.0 g; Ca pantothenate, 1.6 g; folic acid, 0.2 g; biotin, 0.02 g; vitamin B_{12}, 0.001 g; retinyl acetate, 4.0×10^5 units; ergocalciferol, 1.6×10^5 units; menadione, 0.075 g; glucose, 992.5 g.

TABLE 2

Fatty Acid Composition of Dietary Oils

Fatty acid	SO	FO (MO/SO = 9:1)
	%	
14:0	0.1	7.3
15:0	ND[a]	0.4
16:0	10.8	16.7
16:1	0.2	9.9
18:0	3.6	4.5
18:1	20.5	14.8
18:2n-6	55.7	10.7
18:3n-3	7.3	3.8
18:4n-3	ND	4.2
20:5n-3	ND	12.6
22:1	ND	1.4
22:5n-3	ND	1.6
22:6n-3	ND	8.8
Total PUFA	63.0	41.7
n-3/n-6	0.13	2.9
PI[b]	70.3	131.7
Peroxide value[c]	0.75	2.9

[a]Not detected. Abbreviation: PUFA, polyunsaturated fatty acids; see Table 1 for other abbreviations.

[b]Peroxidizability index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

[c]Peroxide values were determined according to the AOAC method (18).

Experimental Animal Management Division, National Institute of Health, Seoul, Korea) were divided into four groups of eight animals each and fed the four respective diets for eight weeks.

Sample preparation. At the end of the feeding period, the rats were fasted for 16 h before sacrifice. Under ether anesthesia, blood was drawn by heart puncture and plasma was prepared with heparin. Liver was immediately removed, washed in saline and rapidly frozen in liquid nitrogen. Plasma and liver was stored at −60°C until analysis.

Chemical assays. Thiobarbituric acid reactive substances (TBARS) in plasma and liver were measured by the methods of Yagi (19) and Hu *et al.* (7), respectively, using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co.) as standard. Plasma uric acid (20), and blood and liver blood glutathione (GSH) (21) were enzymatically assayed. Liver vitamin E was determined according to method of Kayden *et al.* (22). Ten percent liver homogenate in saline was saponified with KOH in the presence of 2% pyrogallol and extracted with hexane. Total tocopherol in the hexane phase was reacted with ferric chloride and dipyridyl. The optical density was measured at 520 nm (22). Vitamin C was extracted from the liver with 5% metaphosphoric acid and measured at 540 nm by the hydrazine method.

High-performance liquid chromatographic analysis. Plasma vitamin A and E were extracted with hexane and determined simultaneously by high-performance liquid chromatography (HPLC) (23) using retinyl acetate (ICN Biochemicals, Cleveland, OH) and (±)-D-tocopheryl acetate (Sigma) as internal standards. A C-18 micro-Bondapak (Waters, Milford, MA) 300 × 8 mm stainless-steel column was used and the analysis (Waters HPLC 510) was carried out isocratically with methanol/H_2O (95:5, vol/vol) as mobile phase and using ultraviolet (UV) detection at 280 nm. To analyze liver vitamin A, tissues were lyophilized, extracted with $CHCl_3$ in the presence of butylated hydroxytoluene (BHT) (50 μg/mL), the extracts saponified with 10% ethanolic NaOH, and reextracted with hexane. HPLC conditions for liver samples were identical to those used for plasma, except that UV detection was at 312 nm. Plasma vitamin C was extracted with 5% metaphosphoric acid and analyzed by HPLC using a Lichrosorb-NH_2 column (250 × 4 mm i.d.), phosphate buffer (pH 3.3) as the mobile phase, and dithioerythrol as standard (24).

Lipid analysis. Plasma total lipids were measured by the method of Frings and Dun (25). Total lipids from 0.5 to 1 g of liver were extracted according to Folch *et al.* (26) and analyzed as for plasma. The lipid extract was subjected to thin-layer chromatography on Silica Gel G 60 plates (Merck, Darmstadt, Germany) using the solvent system petroleum ether (b.p. 30–60°C)/diethyl ether/acetic acid (85:15:1, by vol; Ref. 24) to separate phospholipids from triglyceride and cholesterol. Total liver phospholipids were methylated with BF_3/methanol (14%, w/w) reagent (27). The fatty acid composition of the methyl esters was determined by gas chromatography (Hewlett-Packard, North Hollywood, CA; Model 5890) using a stainless-steel column (2 m × 3 mm i.d.) packed with 20% DEGS on chromosorb W (60–70 mesh) (Gasukuro Kogyo Inc., Tokyo, Japan) and helium as carrier gas at a flow rate of 30 mL/min. The analysis by gas chromatography was performed isothermally at 200°C.

Statistical analysis. Data were analyzed by analysis of variance, and treatment differences were evaluated by

Tukey's test. Correlations between dietary vitamin E levels and biochemical measurements were examined (28).

RESULTS

TBARS levels in tissues. Plasma TBARS levels (per mL) in the FO group reached up to 140% of those in the SO group when rats were fed very low levels (≤3 IU) of vitamin E (Table 3). TBARS levels decreased upon vitamin E supplementation. The FO group had higher levels of plasma TBARS than the SO group fed an equal amount of vitamin E (≤45 IU). This difference became significant when expressed per mg total plasma lipid, which reflected the reduction in total lipids in the FO group (0.83 mg/mL compared with 1.56 mg/mL in the SO group). Supplementation with vitamin E up to ≤209 IU in the FO group decreased the levels of plasma TBARS to those in the SO group. In the liver, TBARS levels (per g tissue) were significantly increased, namely 3.9-fold, 2.6-fold and 2.5-fold in the FO groups with ≤3, ≤45 and ≤209 IU, respectively, when compared to the SO group. When expressed per mg tissue lipid, a similar trend was observed, except that the differences were greater between the FO group with ≤3 IU and the other three groups.

Tissue levels of antioxidants. Plasma vitamin E levels were extremely low in the FO group fed ≤3 IU vitamin E. They were lower in the FO group fed ≤209 IU than in the SO group when expressed as per mL plasma. But they were higher in the FO group than in the SO group at the same level of dietary vitamin E when expressed per mg total plasma lipid, due to the 50% reduction in plasma total lipid in the FO group. Plasma retinol levels were lower in the FO groups at low (≤3 IU/kg) and medium (≤45 IU/kg) levels of dietary vitamin E than in the other two groups. Plasma ascorbic acid levels were significantly lower only in the FO group with ≤3 IU vitamin E, as compared to the SO group. Uric acid levels could not be shown to be different among the groups due to the high variations in measurements. Blood GSH levels in the FO groups were lower than those in the SO group, but the levels were increased in the FO group at ≤209 IU vitamin E when compared with the FO group at ≤3 IU.

In the liver, vitamin E levels (per g tissue) in the FO groups progressively increased with the increase in dietary vitamin E. Vitamin E levels in the SO group were higher than in the FO group at ≤3 IU, but lower than in the other two FO groups. When expressed in per mg liver lipid, the vitamin E level increased only in the FO group at ≤209 IU dietary vitamin E. The hepatic levels of retinol, vitamin C and GSH were not significantly different among the groups.

Correlations of dietary vitamin E with other measured parameters. On the basis of the data obtained on the FO groups (Table 3), correlations of the dietary vitamin E level with various other measurements are presented in Table 4. Dietary vitamin E levels correlated negatively with plasma TBARS ($r = -0.78$, $P < 0.01$) and positively with plasma vitamin E ($r = 0.71$, $P < 0.01$), retinol ($r = 0.53$, $P < 0.05$), vitamin C ($r = 0.64$, $P < 0.05$) and blood GSH (0.56, $P < 0.05$). Consequently, plasma TBARS levels were negatively correlated with plasma vitamin E and blood GSH. Among the plasma and blood antioxidants, fairly strong correlations were found between retinol and vitamin C ($r = 0.64$, $P < 0.05$), and between vitamin C and

TABLE 3

Levels of TBARS, Vitamin E and Other Physiological Antioxidants in Plasma, Blood and Liver of Rats Fed Diets Containing SO and MO + SO (9:1) Mix at Three Levels of Vitamin E[a]

	Vitamin E (IU/kg)			
	≤45	3	45	209
Measurements	SO diet	FO (MO/SO = 9:1) diets		
Plasma				
TBARS[b] (MDA nmoles/mL)	3.62 ± 0.25^{c,e}	5.08 ± 0.41^{d}	4.18 ± 0.47^{c,d}	3.34 ± 0.11^{e}
(MDA nmoles/mg lipid)	2.32 ± 0.22^{c}	4.64 ± 0.43^{d}	4.99 ± 0.30^{d}	4.10 ± 0.13^{e}
Vitamin E (μg/mL)	5.01 ± 0.51^{c}	0.25 ± 0.15^{d}	4.26 ± 0.52^{c,e}	3.33 ± 0.34^{e}
(μg/mg lipid)	3.21 ± 0.26^{c}	0.23 ± 0.04^{d}	5.13 ± 0.71^{e}	4.02 ± 0.69^{c,e}
Retinol (μg/mL)	1.55 ± 0.39^{c}	0.62 ± 0.31^{d}	0.94 ± 0.43^{d}	1.44 ± 0.55^{c}
Ascorbic acid (μg/mL)	7.9 ± 1.3^{c}	5.1 ± 0.2^{d}	6.6 ± 0.4^{c}	7.0 ± 1.9^{c}
Uric acid (μg/mL)	8.0 ± 2.7^{NS}	4.5 ± 3.2	5.7 ± 2.2	12.0 ± 10.8
Blood				
GSH (μmoles/mL)	0.85 ± 0.06^{c}	0.50 ± 0.05^{d}	0.65 ± 0.11^{d,e}	0.68 ± 0.03^{e}
Liver				
TBARS[b] (MDA nmoles/g wet wt)	147 ± 15^{c}	571 ± 40^{d}	380 ± 70^{e}	371 ± 34^{e}
(MDA nmoles/mg lipid)	2.33 ± 0.24^{c}	11.90 ± 0.83^{d}	5.59 ± 1.03^{e}	5.46 ± 0.50^{e}
Vitamin E (μg/g wet wt)	25.6 ± 1.8^{c}	22.5 ± 2.5^{d}	29.6 ± 3.5^{e}	35.8 ± 3.2^{f}
(μg/mg lipid)	0.41 ± 0.03^{c}	0.47 ± 0.04^{c}	0.44 ± 0.05^{c}	0.53 ± 0.04^{d}
Retinol (μg/g dry wt)	764 ± 62^{NS}	633 ± 54	647 ± 92	692 ± 104
Ascorbic acid (μg/g wet wt)	42 ± 6^{NS}	35 ± 7	433 ± 5	36 ± 3
GSH (μmoles/g wet wt)	3.59 ± 0.35^{NS}	3.57 ± 0.42	2.89 ± 0.53	3.00 ± 0.48

[a]All values (superscript letters, c–f) are means ± SD. Values with different superscript letters in the same row are significantly different from each other at $P < 0.05$; NS, not significantly different from the other values in the same row. Abbreviations: TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; MDA, malonaldehyde; see Table 1 for other abbreviations.

[b]Levels of plasma (per mL or per mg lipid) and liver (per g wet wt) TBARS are expressed in terms of nmoles MDA, which were calculated from the relative fluorescence intensity measured at 515 nm excitation and 553 nm emission and from the absorbance at 532 nm, respectively, using 1.1.3.3-tetraethoxypropane as an external standard.

TABLE 4

Correlations Between the Levels of Dietary Vitamin E and Plasma and Blood Measurements in Rats Fed FO (MO/SO = 9:1)[a]

	Plasma					Blood
	TBARS[b]	Vitamin E[b]	Retinol	Vitamin C	Uric acid	GSH
Log dietary vitamin E	−0.78[d]	0.71[d]	0.53[c]	0.64[c]	0.37	0.56[c]
Plasma						
TBARS[b]		−0.53[c]	−0.38	−0.46	−0.23	−0.50[c]
Vitamin E[b]			0.35	0.16	0.19	0.23
Retinol				0.64[c]	0.45[c]	0.45[c]
Vitamin C					0.72[c]	0.60[c]
Uric acid						0.07

[a]Values represent correlation coefficients analyzed from seventeen pairs of observations except those including plasma vitamin C which were from eleven pairs. The values with nonitalic superscript letters c and d have P values of less than 0.05 and 0.01, respectively. See Tables 1 and 3 for abbreviations.
[b]Calculated as per mL plasma.

TABLE 5

Correlations Between the Levels of Dietary Vitamin E and Liver Vitamin E, TBARS and Retinol in Rats Fed FO (MO/SO = 9:1)[a]

	Liver		
	Vitamin E	TBARS	Retinol
Log dietary vitamin E	0.24	−0.57[b]	0.01
Liver vitamin E		0.05	0.39

[a]Values represent correlation coefficients analyzed from seventeen pairs of observations; superscript letter b indicates a P value of less than 0.05. See Tables 1 and 3 for abbreviations.

uric acid ($r = 0.72$, $P < 0.05$) and GSH ($r = 0.60$, $P < 0.05$). Weak correlations were found between retinol and uric acid and GSH. In the liver (Table 5), a significant correlation was seen between dietary vitamin E and liver TBARS ($r = -0.57$, $P < 0.05$). Multiple regression analysis revealed that dietary vitamin E was the sole factor exerting influence on TBARS value in plasma ($R^2 = 0.554$) and liver ($R^2 = 0.358$) and that the effects of antioxidants on TBARS levels in tissues were mediated by dietary vitamin E.

Fatty acid composition of liver phospholipids. The fatty acid compositions of liver phospholipids from the SO group and the FO group with ≤45 IU of dietary vitamin E are presented in Table 6. The fatty acid composition was not different among the FO groups as three different dietary levels of vitamin E and reflected the fatty acid composition of the dietary oil fed.

Comparing the fatty acid composition of liver phospholipids in the FO group with that in the SO group, there was an increase in 16:0 fatty acid content and a reduction in 18:0 fatty acid. Arachidonic (20:4n-6) but not linoleic acid (18:2n-6) in the SO group may have been replaced in part by the n-3 polyunsaturated fatty acid (PUFA) 20:5 and 22:6 and partly by the monounsaturated fatty acids, 16:1 and 18:1n-9. In the FO group, there was a small decrease in total PUFA and virtually no change either in the peroxidizability index or in the unsaturation index, but a 4.6-fold increase in n-3/n-6 ratio was observed when compared with the SO group.

TABLE 6

Fatty Acid Composition (%) of Liver Phospholipids from Rats Fed Diets Containing SO and MO + SO (9:1) with ≤45 IU Vitamin E per kg Diet[a]

Fatty acid	SO diet	FO (MO/SO = 9:1) diets
14:0	0.2 ± 0.0	0.2 ± 0.1
16:0	19.3 ± 1.4	27.7 ± 3.0[b]
16:1	trace	1.4 ± 0.5[b]
18:0	31.5 ± 3.4	23.7 ± 1.0[b]
18:1n-9	3.9 ± 2.1	9.4 ± 0.9[b]
18:2n-6	10.5 ± 2.2	9.0 ± 1.3
18:3n-3	0.2 ± 0.1	0.1 ± 0.1
20:4n-6	28.8 ± 3.6	15.4 ± 2.2[b]
20:5n-3	trace	3.8 ± 2.3[b]
22:0	0.4 ± 0.1	0.7 ± 0.3
22:5n-3	0.2 ± 0.1	0.7 ± 0.5
22:6n-3	3.9 ± 1.1	7.8 ± 3.5[b]
Total PUFA	43.6	36.8
Total n-3 PUFA	4.3	12.6
n-3/n-6	0.12	0.56
PI[c]	117	112
UI[d]	165	160

[a]All values are means ± SD from five to six rats. Values with nonitalic superscript letter b are significantly different from the SO group at $P < 0.05$. Abbreviations in Tables 1 and 2.
[c]Peroxidizibility index is the same as described in Table 2.
[d]Unsaturation index represents the sum of percentages of individual unsaturated fatty acids × number of double bonds.

DISCUSSION

Our study showed that dietary FO increases lipid peroxide levels. The accumulation of lipid peroxide is more pronounced in liver than in plasma. One of the reasons for this difference may be the decreased plasma lipid levels resulting from increased dietary n-3 fatty acid intake (2,29). Lipid content of the liver decreased (30) little or not at all (2,29), or even increased (31). Another reason for increased TBARS levels in liver, even at higher levels of dietary vitamin E, may be that fatty acid peroxides from dietary FO contain a considerable amount of lipid peroxides (2.90 mEq/kg *vs.* 0.75 mEq/kg in SO). The lipid peroxides accumulate and are not secreted into plasma lipoproteins. The increase in lipid peroxide content in the liver of rats fed FO seems to be related to the tissue fatty acid composition as tissue TBARS levels increased parallel with increasing n-3 PUFA content when compared with n-6 PUFA content in tissue phospholipids. Our results showing liver TBARS accumulation in rats fed a FO-supplemented diet and an excess amount (≤209 IU) of

vitamin E is consistent with results described by others (5,6).

TBARS levels in the plasma and in the liver of rats fed FO showed an inverse relation to log dietary vitamin E levels, although the negative correlation of plasma TBARS levels with dietary vitamin E levels was stronger ($r = 0.78$) than that of liver TBARS ($r \approx -0.57$) (Tables 4 and 5). There was a moderate correlation between plasma TBARS with plasma vitamin E ($r = -0.53$, $P < 0.05$) (Table 4), while there was no correlation between liver TBARS and liver vitamin E (Table 5). Leibovitz *et al.* (8) reported a strong correlation between liver TBARS and liver vitamin E, which is not in agreement with our data. This discrepancy may be due in part to differences in experimental conditions, such as the age of rats and differences in the FO used. Rats used in this study were 6-weeks-old and weighed over 150 g, *i.e.*, the animals were older than those used by Leibovitz *et al.* (8). Our rats also maintained substantial liver vitamin E levels even after 8 wk of very low vitamin E (≤3 IU) intake. Another reason could be possible differences in intake of lipid peroxides present in the FO. This points to the practical problems of feeding FO since FO used in most animal experiments have been reported to contain lipid peroxides at levels of 6–22 mEq/kg (17). Some oil products also contain oxidative polymeric materials, although various antioxidants were included (32).

In the present study, we observed not only effects of dietary vitamin E on the status of various antioxidants but also an interrelationship with plasma antioxidant status. Dietary vitamin E showed positive correlations with the status of plasma retinol and vitamin C and blood GSH as well as plasma vitamin E (Table 4). Positive correlations were also found between plasma and blood antioxidants (Table 4). Similar observations have been reported by other investigators (33–38), but these earlier studies were limited to the interactions of a few selected antioxidants. Vitamin E deficiency has been shown to reduce plasma and liver vitamin A levels in rats (33) but not in chickens (34). It is of significance that in the present study vitamin A status was affected by a broad range of vitamin E intake levels. Retinol is known to act as an antioxidant, but its *in vivo* role has not been emphasized as much as that of β-carotene. Tom *et al.* (35) have provided evidence that vitamin A deficiency significantly increased lipid peroxidation in rat lung microsomes, whereas an excess of dietary vitamin A decreased lipid peroxidation. Therefore, retinol may have worked against oxidation in rats fed the FO diet.

Although significant positive correlations were found between plasma retinol, vitamin C, uric acid and blood GSH, the levels of vitamin C, uric acid and GSH were not correlated with plasma vitamin E levels. The roles of GSH and vitamin E in antioxidation have been recognized in earlier studies on liver nuclear membranes (39), microsomes (40) and platelets (41). Regardless of the mechanisms of GSH action, it appears critical to maintain a sufficient amount of total glutathione and to efficiently recycle the oxidized form (GSSG) to the reduced form (GSH). In rat heart and liver (36,37), vitamin A deficiency has been shown to increase the total glutathione pool, whereas in rat lung microsomes (35), both a deficiency and an excess of vitamin A reduced the glutathione content. Reduction of GSSG to GSH occurs by the action of glutathione reductase (GR), which is not influenced by dietary factors (42). NADPH, the reductant in the GR reaction, is supplied mainly by the glucose-6-phosphate dehydrogenase (G6PDH) reaction and appears to determine recycling (38). The increase in erythrocyte NADP was associated with a reduced GSH/GSSG ratio (38). FO decreases G6PDH activity (29,43), which in turn slows down the formation of NADPH. This may explain the low level of GSH in the FO group fed ≤209 IU vitamin E, in spite of the sparing action of vitamin E in this group.

Plasma vitamin C levels were significantly decreased in the FO group fed ≤3 IU vitamin E despite the fact that rats can synthesize vitamin C *in vivo*. Studies by Ingold's group (44) have shown the antioxidant activity of vitamin C in cooperation with vitamin E in an aqueous solution containing phospholipid liposomes, but could not demonstrate its sparing effect *in vivo* using guinea pigs (45). More recently, however, Igarashi *et al.* (46) have observed the synergistic action of vitamin E and C in mutant rats, called osteogenic disorder Shionogi (ODS) rats (47), which are genetically incapable of synthesizing vitamin C. In humans, who need relatively large amounts of vitamin C, the body status of vitamin C is more likely to be disturbed under conditions of oxidative stress brought about by the intake of FO and/or an insufficient amount of vitamin E.

The present study demonstrates an increase in vitamin E requirements with FO feeding not only to prevent enhanced lipid peroxidation but also to maintain an appropriate status of other antioxidants. More studies are needed to determine what an adequate ratio of dietary vitamin E to FO should be.

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Effect of Maternal Dietary Arachidonic or Linoleic Acid on Rat Pup Fatty Acid Profiles

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Rapidly growing neonatal mammals accrete relatively large quantities of long chain ($\geq C_{20}$) polyunsaturated fatty acids (LCP) in membrane phospholipids. We have examined accumulation of ω6 LCP in suckling neonatal rat pups during the first 14 d of life when their dams received essential fatty acids in the form of triglycerides containing linoleic acid or arachidonic acid. Dietary levels of these fatty acids were either 1 or 5% of total dietary fatty acids. The fatty acid profile of pup stomach contents (composed solely of the dams' milk) and plasma lipids, as well as liver and brain phospholipids, were determined. Stomach linoleic and arachidonic acid levels reflected the diet of the dams. Pup plasma and liver arachidonic acid levels increased progressively from the group receiving 1% linoleic acid to 5% linoleic acid and from 1% arachidonic acid to 5% arachidonic acid. Interestingly, brain phosphatidylethanolamine and phosphatidylcholine arachidonic acid levels were more stable than plasma or liver levels. These results suggest that the brain may be capable of either selective transport of ω6 LCP or chain elongation/desaturation of linoleic acid. These data indicate that care must be exercised when adding LCP to infant formula since widely divergent accretion rates of arachidonic acid may occur in various tissues. ***Lipids 29*****, 53–59 (1994).**

Polyunsaturated fatty acids of the ω6 family are essential nutrients, as clearly delineated by Burr and Burr (1,2). Deficiencies of these fatty acids result in impaired growth and reproductive performance, skin lesions and inappropriate activity of the immune system (3). In newborn, rapidly growing mammals the requirements for essential fatty acids are particularly high. For example, during the last three months of pregnancy and first 18 mon of life, humans rapidly accumulate fatty acids in the central nervous system in a process termed the brain growth spurt (4–6). Both ω3 and ω6 long chain polyunsaturated fatty acids (LCP, $\geq C_{20}$) are accumulated, although ω6 accretion rates are higher than ω3 rates (5,6). Microsomal desaturation and chain elongation pathways may be submaximal during this time period, thus, optimal rates of LCP accretion may be dependent on appropriate supplies of preformed LCP or on appropriate dietary levels of precursor ω3 and ω6 fatty acids.

Human milk contains 10–15% linoleic acid and 0.5–1.0% arachidonic acid, the chain elongation/desaturation product of linoleic acid (7). Similarly, both α-linolenic acid and ω3 LCP are present in human milk. Clandinin *et al.* (8) calculated that the levels of ω6 and ω3 LCP in breast milk are sufficient to meet the needs of rapidly growing infants. Currently, infant formulas employ vegetable oils or a mixture of vegetable and animal oils to meet the essential fatty acid requirements of the infant, as defined by numerous pediatric advisory organizations and regulatory agencies (9). In general, minimal linoleic acid levels are set between 300 and 500 mg per 100 kcal of formula. The oils employed in formulas do not contain LCP of either the ω3 or ω6 class. Although breast-fed infants have relatively constant circulating levels of ω3 and ω6 LCP, some reports document that formula-fed preterm (10–12) and term (13) infants undergo a time-related decrease in circulating ω3 and ω6 LCP levels. Concern that low ω3 LCP levels may result in reduced visual acuity in preterm infants has led several groups to examine the addition of fish oils to preterm formulas (10,14,15). During a long-term feeding study, Carlson *et al.* (10) noted that although the ω3 LCP status of preterm infants fed marine oil-supplemented formula was improved, circulating arachidonic acid levels fell significantly. Thus, addition of both ω3 and ω6 LCP to formula (mimicking breast milk) may be prudent.

Sources of ω3 LCP are readily available from a variety of marine oil sources (16). However, economically viable sources of ω6 LCP are much more difficult to develop. In the present study we have employed a suckling rat model previously described by Yeh *et al.* (17) to examine tissue ω6 LCP accretion, comparing an oil source rich in linoleic acid (peanut oil) to a novel triglyceride source of arachidonic acid. Diets containing 1 or 5% linoleic or arachidonic acid were fed to dams and the resulting fatty acids quantitated in their milk. The incorporation of ω6 LCP in the pups was determined in liver and brain phospholipids as well as plasma lipids.

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in a room controlled for temperature (21 ± 1°C), humidity (40–50%) and light (lights on 0600–1800 h). The rats were housed in suspended wire mesh cages before and during the mating period. They received a standard rodent chow (Purina Rat Chow; Ralston Purina, St. Louis, MO). The female rats were mated (monogamous mating one male/one female) for a two-week period. After the mating period, the female rats were divided into four groups (five dams/group). The pregnant rats were housed individually in plastic cages with ALPHA-dri™ bedding and given free access to food and water.

Newborn pups were delivered naturally and were weighed within 24 h of birth. Semi-purified diets containing either 1 or 5% linoleic acid, or 1 or 5% arachidonic acid were introduced to the dams on postpartum day 2. The diets containing arachidonic acid were prepared and stored at −70°C under argon. The diets containing linoleic acid were prepared and stored at 5°C. All four diets contained 22% casein, 1% cellulose, 4% Bernhart-Tomarelli Salt Mixture, 1% AIN-76 vitamin mix (both salt mix and vitamin mix from United States Biochemical Corp., Cleveland, OH), 0.1% choline chloride, 56.9% dextrose and 15% fat. Oil sources for the diets were hydrogenated

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Abbreviations: DHA, docosahexaenoic; LCP, long chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

TABLE 1

Fatty Acid Profile of Dietary Fats

Carbon number	Diet A[a]: low linoleic acid	Diet B[b] high linoleic acid	Diet C[c] low arachidonic acid	Diet D[d] high arachidonic acid
	(percentage by weight)			
6:0	0.6	0.5	—	—
8:0	7.4	6.4	6.2	4.3
10:0	5.6	4.8	5.3	4.0
12:0	44.2	38.0	42.6	31.7
14:0	16.7	14.3	18.7	15.8
16:0	8.8	8.7	10.6	11.8
16:1	—	—	0.5	2.7
18:0	11.7	10.5	11.9	9.9
18:1ω9	2.9	10.4	0.9	2.2
18:1ω7	—	—	1.6	7.9
18:2ω6	0.9	4.4	0.1	0.4
18:3ω6	—	—	0.1	0.6
18:3ω3	0.6	0.5	—	—
20:0	0.8	1.6	0.2	0.2
20:2ω6	—	—	—	0.1
20:3ω6	—	—	—	0.2
20:4ω6	—	—	1.0	4.8
22:6ω3	—	—	—	0.5

[a]Diet A: 3.4% peanut oil, 96.6% hydrogenated coconut oil.
[b]Diet B: 17.0% peanut oil, 83.0% hydrogenated coconut oil.
[c]Diet C: 5.0% arachidonic acid-containing oil, 95.0% hydrogenated coconut oil.
[d]Diet D: 25.0% arachidonic acid-containing oil, 75.0% hydrogenated coconut oil.

coconut oil and either peanut oil (both from ICN Biomedicals, Inc., Aurora, OH) or an arachidonic acid-rich triglyceride. Table 1 provides the fatty acid profiles of the diets.

On postpartum day 0, the litter size (number live/number dead) was determined. On day 7, the pups were weighed, sexed and culled to four males and four females per litter. On day 15, the pups were weighed, anesthetized with an intraperitoneal injection of Nembutal™ (5 mg/100 g body weight) and blood obtained by cardiac puncture with a 1-mL syringe containing ethylenediaminetetraacetate sodium. Approximately 1.0 mL of blood was routinely obtained per pup; plasma was collected and pooled from each litter. The brain, liver and stomach contents were obtained from one male rat per litter. The brain and liver were immediately rinsed in ice-cold saline. All samples were stored at −70°C until lipid analysis.

Lipid extraction and analyses. Lipids were extracted from tissues and plasma according to the procedure of Folch *et al.* (18). For analyses of plasma fatty acids, a 1-mL aliquot was added to 20 mL of a chloroform/methanol (2:1, vol/vol) mixture for lipid extraction. The tissues were weighed, distilled water was added (10 mL for every gram of tissue) and homogenized. For analyses of phospholipid fatty acids, an aliquot of the homogenate (1 mL) was added to 20 mL of a chloroform/methanol (2:1, vol/vol) mixture for lipid extraction.

Phospholipid classes were separated by thin-layer chromatography using LK6 silica plates and a solvent system consisting of chloroform/methanol/petroleum ether (b.p. 30–60°C)/acetic acid/boric acid (40:20:30:10:1.8, vol/vol/vol/vol/wt) (19). Santoquin™ was added to the methanol as antioxidant.

Lipids were methylated and extracted using the method of Morrison and Smith (20). Fatty acid methyl esters were analyzed by gas chromatography (Hewlett-Packard Model 5890 GC interfaced with an HP 3365 Chemstation; Palo Alto, CA) using a capillary column (Omegawax, 30 m, 0.32 mm i.d.; Supelco, Bellefonte, PA) and flame-ionization detection. The following operating parameters were used: column temperature, 200°C, isothermal; injector, 230°C; detector, 250°C; split ratio, 65:1; carrier gas, helium. Appropriate standards were analyzed to determine retention times and response factors (Nu Chek-Prep, Inc., Elysian, MN). Fatty acid composition was expressed as the weight percent of the total fatty acids.

Statistics. Data were analyzed using the Student-Newman-Keuls test ($P = 0.05$) with a one-way completely randomized design.

RESULTS

Growth. The weight gain of the pups from postpartum day 1 to day 15 was not statistically different between groups (mean ± SEM;1% linoleic acid, 23.5 ± 0.8 g; 5% linoleic, 26.2 ± 0.5 g; 1% arachidonic, 22.2 ± 2.3 g; 5% arachidonic, 21.3 ± 2.9 g). Weights of livers and brains were also similar for all groups (data not shown). Diet had no statistically significant effect on the weights of the dams at postpartum day 15.

Stomach contents. Stomach content of the pups is a reflection of maternal milk composition. As expected, the fatty acid profile of the pup stomach contents varied in several fatty acids (Table 2). The linoleic acid content from pups whose dams received 5% dietary linoleic acid was significantly elevated compared to the other groups. Similarly, the high-dose arachidonic acid group had elevated stomach arachidonate levels compared to the

TABLE 2

Effect of Maternal Dietary Fat Composition on Suckling Pup Stomach Content Fatty Acid Composition[a]

Fatty acid	Group A: low linoleic acid	Group B: high linoleic acid	Group C: low arachidonic acid	Group D: high arachidonic acid
		(percentage by weight)		
8:0	1.8 ± 0.61	2.5 ± 0.57	2.0 ± 0.99	2.1 ± 0.48
10:0	9.0 ± 1.04	9.7 ± 1.09	9.3 ± 1.42	10.6 ± 0.35
12:0	26.7 ± 0.89	26.1 ± 0.41	26.2 ± 0.91	24.7 ± 0.81
14:0	19.2 ± 0.41	16.7 ± 0.56	18.0 ± 1.28	17.1 ± 0.68
16:0	24.5 ± 1.46	20.1 ± 0.68	23.9 ± 2.13	21.1 ± 0.91
16:1ω7	0.5 ± 0.14[b]	0.5 ± 0.12[b]	0.7 ± 0.19[b]	2.0 ± 0.12[c]
18:0	8.8 ± 0.18	9.1 ± 0.63	9.8 ± 1.19	8.5 ± 0.38
18:1ω9	6.5 ± 0.44[b]	10.1 ± 0.32[c]	6.3 ± 0.41[b]	5.5 ± 0.85[b]
18:2ω6	0.8 ± 0.15[b]	2.9 ± 0.12[c]	0.5 ± 0.08[b]	0.7 ± 0.14[b]
20:4ω6	0.3 ± 0.07[b]	0.3 ± 0.02[b]	0.5 ± 0.09[b]	2.2 ± 0.10[c]

[a]Values reported are weight percent and are means ± SEM (n = 4-5).
[b,c]Values not bearing the same superscript letters are different at $P < 0.05$.

other groups. The low-dose arachidonic acid group contained a numerically higher (not statistically significant) arachidonic acid level compared to the linoleic acid groups.

Liver phospholipid levels. Liver phosphatidylcholine (PC) linoleic acid levels increased in a dose-related manner in the groups whose dams received either 1 or 5% linoleic acid (Table 3), although differences were small compared to stomach content levels. Interestingly, the groups receiving dietary arachidonic acid showed a statistically significant dose-related decrease in liver PC linoleic acid levels, even though this was not reflected in the pups' stomach contents. Liver PC arachidonic acid levels increased progressively from the 1 to 5% linoleic acid group and the 1 to 5% arachidonic acid group. Levels of 22:4ω6 were significantly elevated in the high-dose arachidonic acid group compared to the other groups. No significant differences occurred in liver docosahexaenoic acid (DHA) in any of the groups.

Trends similar to the liver PC profiles described above were also noted for liver phosphatidylethanolamine (PE) (Table 3), with the exception that the arachidonic acid response was not as striking in PE as in PC. Additionally, DHA levels fell significantly and in a dose-related manner as ω6 LCP levels increased (DHA level in 1% linoleic acid group, 17.9%; DHA level in 5% arachidonic group, 10.7%; $P < 0.05$).

TABLE 3

Effect of Maternal Dietary Fat Composition on Suckling Pup Liver Phospholipid Fatty Acid Composition[a]

Fatty acid	Group A: low linoleic acid	Group B: high linoleic acid	Group C: low arachidonic acid	Group D: high arachidonic acid
Phosphatidylcholine		(percentage by weight)		
14:0	2.5 ± 0.23[b]	2.2 ± 0.23[b]	2.6 ± 0.20[b]	1.4 ± 0.24[c]
16:0	32.2 ± 0.87	30.6 ± 1.04	32.2 ± 0.48	28.6 ± 1.14
16:1	0.7 ± 0.13	0.4 ± 0.09	0.6 ± 0.09	0.5 ± 0.03
18:0	18.9 ± 0.53[b,c]	21.0 ± 0.67[b]	17.8 ± 0.70[c]	20.7 ± 0.57[b]
18:1ω9	10.4 ± 1.34[b]	8.1 ± 0.87[b,c]	6.7 ± 0.73[c]	3.0 ± 0.14[d]
18:2ω6	5.7 ± 0.31[b]	7.1 ± 0.19[c]	3.3 ± 0.12[d]	1.3 ± 0.06[e]
20:3ω6	2.0 ± 0.09[b]	1.7 ± 0.12[c]	1.1 ± 0.06[d]	0.6 ± 0.03[e]
20:4ω6	11.9 ± 1.12[b]	16.6 ± 0.73[c]	18.8 ± 0.57[c]	27.3 ± 1.12[d]
22:4ω6	0.5 ± 0.05[b]	0.5 ± 0.02[b]	0.8 ± 0.05[b]	3.2 ± 0.48[c]
22:5ω6	2.2 ± 0.19	1.7 ± 0.14	1.4 ± 0.06	1.6 ± 0.49
22:5ω3	0.5 ± 0.09[b]	0.6 ± 0.08[b]	1.1 ± 0.08[c]	1.3 ± 0.10[c]
22:6ω3	8.1 ± 0.44	8.0 ± 0.38	9.8 ± 0.44	7.7 ± 1.04
Phosphatidylethanolamine				
16:0	24.8 ± 0.96[b]	26.0 ± 2.36[b]	31.8 ± 1.94[c]	22.8 ± 0.71[b]
18:0	28.7 ± 1.54	30.6 ± 1.65	31.0 ± 1.77	30.1 ± 2.03
18:1ω9	2.7 ± 0.31[b]	2.3 ± 0.15[b]	2.3 ± 0.15[b]	1.3 ± 0.06[c]
18:2ω6	1.5 ± 0.19[b]	2.0 ± 0.26[b]	1.3 ± 0.33[b]	0.5 ± 0.03[c]
20:4ω6	16.0 ± 0.58[b]	17.5 ± 1.16[b]	13.9 ± 1.48[b]	20.7 ± 0.89[c]
22:4ω6	1.0 ± 0.09[b]	1.1 ± 0.09[b]	0.9 ± 0.11[b]	5.5 ± 0.66[c]
22:5ω6	2.8 ± 0.61	2.7 ± 0.33	1.9 ± 0.45	2.5 ± 0.62
22:5ω3	0.9 ± 0.16[b]	0.9 ± 0.12[b]	0.9 ± 0.15[b]	1.6 ± 0.19[c]
22:6ω3	17.9 ± 1.21[b]	13.0 ± 1.20[c]	10.8 ± 1.96[c]	10.7 ± 1.26[c]

[a]Values reported are weight percent and are means ± SEM (n = 4-5).
[b-e]Values not bearing the same superscript letters are different at $P < 0.05$.

TABLE 4

Effect of Maternal Dietary Fat Composition on Suckling Pup Plasma Lipid Fatty Acid Composition[a]

Fatty acid	Group A: low linoleic acid	Group B: high linoleic acid	Group C: low arachidonic acid	Group D: high arachidonic acid
		(percentage by weight)		
12:0	4.2 ± 0.41[b]	3.4 ± 0.43[b]	3.3 ± 0.13[b]	2.0 ± 0.37[c]
14:0	6.0 ± 0.52[b]	4.6 ± 0.34[c]	4.8 ± 0.12[b]	3.5 ± 0.30[d]
16:0	25.7 ± 0.76[b]	23.4 ± 0.28[c]	24.1 ± 0.91[c]	22.0 ± 0.32[c]
16:1ω7	1.2 ± 0.15[b]	0.6 ± 0.03[c]	1.3 ± 0.15[c]	1.0 ± 0.03[b]
18:0	22.5 ± 1.27	22.5 ± 0.77	22.0 ± 0.30	20.8 ± 0.33
18:1ω9	10.8 ± 1.32[b]	8.4 ± 0.41[b]	9.2 ± 0.88[b]	3.5 ± 0.26[c]
18:1ω7	1.0 ± 0.07[b]	0.8 ± 0.05[b]	1.4 ± 0.13[c]	2.2 ± 0.12[d]
18:2ω6	8.2 ± 0.85[b]	13.9 ± 0.26[c]	5.8 ± 0.49[d]	2.5 ± 0.26[e]
18:3ω6	0.3 ± 0.08	0.4 ± 0.10	0.2 ± 0.10	0.2 ± 0.16
20:3ω6	1.0 ± 0.05[b,c]	1.1 ± 0.04[b]	1.0 ± 0.06[b,c]	0.9 ± 0.02[c]
20:4ω6	10.9 ± 1.73[b]	15.7 ± 0.69[c]	20.1 ± 0.60[d]	36.8 ± 0.98[e]
22:4ω6	0.1 ± 0.08[b]	—	0.5 ± 0.13[c]	1.7 ± 0.07[d]
22:5ω6	0.6 ± 0.15	0.5 ± 0.20	0.2 ± 0.15	0.2 ± 0.15
22:5ω3	0.1 ± 0.08	—	0.1 ± 0.12	—
22:6ω3	3.5 ± 0.59	3.3 ± 0.12	4.1 ± 0.33	2.7 ± 0.28

[a]Values reported are weight percent and are means ± SEM (n = 4-5).
[b-e]Values not bearing the same superscript letters are different at $P < 0.05$.

Plasma lipids. Pup total plasma linoleic acid levels increased in a dose-related manner in the 1 and 5% linoleic acid groups, and progressively decreased in the 1% and 5% arachidonic acid groups (Table 4), similar to the liver PC data. Pup plasma arachidonic acid levels increased more than threefold from the 1% linoleic group to the 5% arachidonic group. Similarly, 22:4ω6 increased in a dose-related manner. No significant alterations occurred in ω3 LCP.

Brain fatty acid levels. In distinct contrast to the liver and plasma data, no significant differences occurred between groups for brain PC or PE levels of arachidonic acid (Table 5). Interestingly, brain PC linoleic acid levels were significantly higher in groups whose dams received dietary linoleic acid compared to the arachidonic acid groups (Table 5). No significant differences were noted in brain PC or PE DHA levels between any of the groups, although a trend to lower DHA levels was found in the high arachidonic acid group.

DISCUSSION

The present study employed maternal dietary alteration as a method of delivering diets of various fatty acid compositions to rapidly growing rat pups. The direct effect of maternal dietary unsaturated fatty acid composition on resultant milk fatty acid profiles has been clearly demonstrated in humans (7) and in rodents (17,21,22). In the present study, we observed a correlation of maternal dietary ω6 fatty acid profile and stomach ω6 fatty acid profile of pups. Interestingly, the levels of expression of ω6 fatty acids in the dams' milk were lower than the dietary levels of supplemented fatty acids, suggesting a concentration gradient from maternal diet and plasma to milk. The efficiency of LCP transfer from the circulation to milk is relatively poor; maternal circulating levels of LCP are much higher than milk LCP levels in both humans (23-25) and rodents (26). Essential fatty acids may be diluted in milk, since nonessential fatty acids may be synthesized *de novo* by the mammary gland, while essential fatty acids must be provided from the circulation.

Liver and plasma arachidonic acid levels in the pups followed an extremely strong dose-related pattern, increasing progressively from the group receiving 1% linoleic acid to the group receiving 5% arachidonic acid. Arachidonic acid content in milk was equivalent in the groups receiving 1 and 5% linoleic acid (milk arachidonic acid levels were 0.3 ± 0.07 wt% and 0.3 ± 0.02 wt%, respectively), while there was a fourfold difference in the milk linoleic acid levels of these groups (Table 2). These data suggest that hepatic chain elongation and desaturation must be active in these young rats. Substrate supply for ω6 LCP synthesis was substantially different, resulting in significant differences in hepatic arachidonic acid levels. Previous studies demonstrated that fatty acid chain elongation and desaturation at the level of the liver is active in young mammals. Hepatic Δ6 desaturase increases rapidly in newborn mice, reaching a maximum at day 7 postpartum followed by a slow decline (27). Hepatic synthesis of arachidonic acid from linoleic acid increases tenfold when fetal piglets are compared to newborn piglets (28,29).

Even though arachidonic acid can be synthesized from linoleic acid, administration of dietary arachidonic acid is clearly a superior means of promoting hepatic LCP accretion. Not only are hepatic phospholipid levels of arachidonic acid elevated in groups directly receiving arachidonic acid (Table 3), but analysis of total liver lipid also indicates a dramatic increase in total arachidonic acid (data not shown). Previous studies in both rats (30) and mice (31) have also demonstrated that dietary arachidonic acid is much more potent in supporting hepatic arachidonate accretion than is linoleic acid. This may be due to relatively limited conversion of linoleic acid to arachidonic acid (32) or relatively rapid oxidation of linoleic acid compared to arachidonic acid (33).

In the present study, dietary arachidonic acid resulted in a significant reduction of hepatic and plasma linoleic acid levels. The effect was dose-related and independent of the level of linoleic acid the pups received (the stomach

TABLE 5

Effect of Maternal Dietary Fat Composition on Suckling Pup Brain Phospholipid Fatty Acid Composition[a]

Fatty acid	Group A: low linoleic acid	Group B: high linoleic acid	Group C: low arachidonic acid	Group D: high arachidonic acid
Phosphatidylcholine		(percentage by weight)		
14:0	2.04 ± 0.53	1.7 ± 0.29	1.9 ± 0.50	1.9 ± 0.42
16:0	49.4 ± 0.78	52.0 ± 1.76	51.5 ± 0.79	48.9 ± 1.68
16:1ω7	1.3 ± 0.47	1.2 ± 0.43	0.9 ± 0.61	1.2 ± 0.32
18:0	11.4 ± 0.91	11.9 ± 0.59	10.8 ± 0.47	15.7 ± 2.53
18:1ω9	15.0 ± 0.78	15.8 ± 1.15	15.6 ± 0.24	12.4 ± 1.08
18:2ω6	0.9 ± 0.09[b]	1.0 ± 0.03[b]	0.6 ± 0.07[c]	0.3 ± 0.03[d]
18:3ω3	0.3 ± 0.04	0.3 ± 0.08	0.2 ± 0.06	0.2 ± 0.06
20:2ω6	0.2 ± 0.01[b]	0.2 ± 0.02[b]	0.1 ± 0.01[c]	0.1 ± 0.02[c]
20:3ω6	0.4 ± 0.20[b]	0.3 ± 0.02[c]	0.3 ± 0.03[c]	0.2 ± 0.02[d]
20:4ω6	6.1 ± 0.50	5.6 ± 0.98	5.7 ± 0.79	6.1 ± 0.45
22:1ω9	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.04	0.,2 ± 0.05
22:4ω6	0.5 ± 0.08	0.5 ± 0.08	0.5 ± 0.06	0.7 ± 0.07
22:5ω6	0.4 ± 0.03	0.3 ± 0.05	0.2 ± 0.06	0.3 ± 0.04
22:6ω3	2.1 ± 0.17	1.6 ± 0.40	1.7 ± 0.45	1.4 ± 0.28
Phosphatidylethanolamine				
14:0	0.5 ± 0.06	0.6 ± 0.27	0.8 ± 0.13	0.5 ± 0.16
16:0	9.6 ± 0.87	9.8 ± 1.14	8.8 ± 0.22	9.7 ± 0.77
16:1ω7	0.7 ± 0.04	0.8 ± 0.16	0.8 ± 0.10	0.7 ± 0.17
18:0	29.8 ± 3.25	29.8 ± 2.02	30.3 ± 2.16	31.4 ± 0.83
18:1ω9	7.1 ± 0.09	6.3 ± 0.46	6.6 ± 0.41	6.6 ± 0.22
18:2ω6	0.7 ± 0.24	0.6 ± 0.08	0.4 ± 0.16	0.5 ± 0.27
18:3ω3	0.6 ± 0.26	0.6 ± 0.23	0.8 ± 0.37	0.8 ± 0.17
20:2ω6	0.4 ± 0.05	0.5 ± 0.11	0.4 ± 0.04	0.2 ± 0.03
20:3ω6	1.9 ± 0.32	1.6 ± 0.15	1.2 ± 0.28	0.8 ± 0.20
20:4ω6	15.1 ± 1.77	14.0 ± 1.16	15.5 ± 0.66	16.7 ± 0.14
22:1ω9	0.5 ± 0.07	0.7 ± 0.13	0.7 ± 0.19	0.6 ± 0.16
22:4ω6	4.4 ± 0.44[b]	4.5 ± 0.67[b]	5.1 ± 0.25[b]	6.6 ± 0.13[c]
22:5ω6	2.4 ± 0.28	2.2 ± 0.27	2.2 ± 0.19	2.8 ± 0.27
22:6ω3	15.0 ± 2.36	13.5 ± 1.97	15.1 ± 1.37	12.1 ± 1.26

[a]Values reported are weight percent and are means ± SEM (n = 4–5).
[b-d]Values not bearing the same superscript letters are different at $P < 0.05$.

linoleic acid contents of pups in the 1% linoleic group, 1% arachidonic group and 5% arachidonic group were equivalent). Thus, arachidonic acid may replace linoleic acid for incorporation into phospholipid membranes. A similar observation was made by Mohrhauer and Holman (30) in young rats fed increasing doses of arachidonic acid. As the dietary level of arachidonic acid was increased, liver linoleic acid levels decreased. Seyberth *et al.* (34) also reported decreased plasma levels of linoleic acid following ethyl arachidonate administration to adult humans.

Brain PE and PC arachidonic acid levels were similar in all groups. This is surprising in light of a threefold difference in plasma arachidonic acid concentration between groups. This highly protected brain arachidonic acid profile may be achieved in one of two ways: (i) by active transport of arachidonic acid from the circulation or (ii) through chain elongation/desaturation of linoleic acid in brain. Sinclair (35) demonstrated rapid uptake of [^{14}C]arachidonic acid by suckling rat pup brain and incorporation into phospholipids. When radiolabeled linoleic acid was administered, accumulation of phospholipid arachidonic acid was approximately three times lower than when radiolabeled arachidonic acid was administered. The preferential transport of LCP *vs.* C_{18} precursors was supported by similar data comparing α-linolenic acid transport to DHA transport in suckling rats (36). The second possibility, existence of chain elongation/desaturation pathways in the brain of developing animals, must also be considered. The level of brain Δ6 desaturase is relatively high at birth and falls rapidly during the first three weeks of life in the suckling mouse (27). In contrast, synthesis rates of chain elongation/desaturation products in the piglet brain are similar at birth and in older animals (28,29); however, these rates are less than 10% of the hepatic synthesis rates. With only the relative transport and synthesis data outlined above, no firm conclusions can be drawn concerning the mechanism of arachidonic acid accretion that we have observed.

The data of Mohrhauer and Holman (37) demonstrated that brain arachidonic acid levels could be altered in a dose-related manner by orally administered ethyl esters of ω-6 fatty acids; ethyl arachidonate was more potent and yielded higher maximal arachidonic acid levels than ethyl linoleate. This study employed ethyl esters as the sole source of lipid in weanling rats studied for 100 d. The present study, employing the rodent dam to provide nutrition to rapidly growing pups, allows for less controlled fatty acid delivery. All pups received a basal level of

arachidonic acid from their mothers' milk. The level of arachidonic acid received in the linoleic acid groups (0.3% of fatty acids, or about 0.2% of calories) would be sufficient to promote modest brain arachidonic acid accretion if placed in the context of the Mohrhauer and Holman (37) dose levels. The previous experiment determined total brain fatty acid profiles, while the present results report brain PC and PE levels. We have also measured brain total fatty acid profiles (data not shown) and found no significant differences in arachidonic acid levels between groups. Therefore, the discrepancies between the previous results and current data cannot be due to a brain lipid fraction response occurring independently of PC and PE.

The possibility that the C_{18} precursors of LCP found in infant formula may be sufficient for needs during infancy is an area of current investigation (38). Our results demonstrate that modest levels of dietary linoleic acid can support normal brain arachidonic acid accretion, even though plasma and hepatic arachidonic acid levels may appear to be low. However, the current study was conducted with a small number of animals (limiting its statistical power) and for a relatively short duration. It is conceivable that a longer study with more animals may have demonstrated statistically different brain fatty acid levels.

Arbuckle and Innis (39) have made an observation similar to ours in the retina of formula-fed piglets when studying ω3 status. Animals receiving formulas relatively low in α-linolenic acid had retinal DHA levels equivalent to animals receiving a fish oil supplemented diet, while higher levels of dietary α-linolenic acid produced both brain and retinal levels of DHA similar to sow-fed controls. Although these results may suggest that C_{18} precursors of LCP may be useful for supplying the infant with LCP, the optimal means of supplying ω3 and ω6 essential fatty acids appears to be the direct addition of LCP to formula. Numerous studies in term and preterm infants have demonstrated reduced levels of ω3 and ω6 LCP in the circulation of formula-fed infants compared to infants receiving human milk (10–13). The addition of ω3 LCP to preterm formula has resulted in improved visual acuity and has evoked visual potential (40,41). Unfortunately, feeding of a formula containing ω3 LCP alone suppresses circulating arachidonic acid levels and may lead to slower growth and altered indices of mental development (42,43). Addition of both ω3 and ω6 LCP thus appears to be appropriate.

The results of the current experiment suggest that linoleic acid is capable of supporting normal arachidonic acid brain accretion. However, the diets employed in this study were virtually free of ω3 fatty acids. Inclusion of ω3 LCP in the diets of rodent dams appears to inhibit chain elongation/desaturation of linoleic in the pups (44). Thus, animals receiving ω3 LCP plus linoleic acid have lower brain ω6 LCP accretion than animals receiving dietary ω3 and ω6 LCP (44). The arachidonic acid source employed in the present study may be appropriate for addition to infant formula, but should be added in the presence of a source of ω3 LCP.

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Abnormal Plasma Lipids of Patients with *Retinitis pigmentosa*[1]

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***Retinitis pigmentosa* (RP) is a hereditary retinal degeneration of unknown etiology, resulting in progressive night blindness, loss of peripheral vision, abnormal retinal pigmentation and reduced electroretinographic response. Docosahexaenoic acid (22:6ω3) is found in high concentration in the rod outer segment membranes of the retina. Previous reports of low 22:6ω3 in blood lipids or phospholipids in RP patients prompted us to evaluate the complete fatty acid (FA) profiles of plasma phospholipids (PL), cholesteryl esters, triglycerides (TG) and nonesterified fatty acids (NEFA) in ten patients with RP. In the PL fraction, we found significantly depressed levels of 22:6ω3, 22:5ω3, total ω3, 22:5ω6, 22:4ω6 and total ω6 polyunsaturated FA (PUFA), and elevated total saturated acids. Plasma TG showed normal levels of PUFA, normal total saturated FA and total monounsaturated FA. The NEFA fraction showed significant elevation in total saturated FA with depressed total ω6 PUFA. Evidence is accumulating that RP is associated with abnormal PUFA and lipid metabolism.**

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Retinitis pigmentosa (RP) is a hereditary retinal degeneration with symptoms of reduced night vision and loss of peripheral visual field, beginning in young adults. By an unknown mechanism, the photoreceptors and retinal pigment epithelium (RPE) are injured with resulting reduced light sensitivity, loss of pigmentation of the RPE and pigment deposition in the sensory retina (1). Familial inheritance of retinal epithlium may be autosomal dominant, autosomal recessive or X-linked, but a "simplex" presentation with no known affected family members, or "multiplex" with two or more affected siblings without other affected relatives is also common (2). Prevalence in the general population is approximately 1 in 4000 (3).

The polyunsaturated fatty acid (PUFA) 22:6ω3, docosahexaenoic acid (DHA), accounts for about 50% of the fatty acid (FA) content of the rod outer segments (ROS) of the vertebrate retina (4-6) and is highly conserved even after severe dietary deprivation of ω3 FA (4,6). Physiologic needs, which may explain this high content of PUFA in the ROS, remain speculative, but enhanced membrane fluidity has been correlated with high 22:6ω3 content in human retinoblastoma cells in tissue culture (7). Using radioactive molecular labelling in an animal model, the recycling of 22:6ω3 has been demonstrated from ROS to the RPE and back to the ROS across the interphotoreceptor matrix (8).

Converse and colleagues (9) in 1983 discovered low plasma DHA in patients from three unrelated Scottish families with RP. Since their report, several groups have described abnormal DHA levels in blood phospholipids (PL) or total lipids of patients with RP, most finding diminished levels (10-12), but in one report, elevated DHA was found in patients from a single autosomal dominant pedigree (13). Lipid measurements for patients in these studies were compared to unaffected family members.

We have made a comprehensive analysis of FA composition in plasma lipids from ten patients with RP. Four major lipid fractions were isolated, and the content of 25 FA was measured in PL, triglycerides (TG), nonesterified fatty acids (NEFA) and cholesteryl esters (CE). The compositions for RP patients were compared to normal values for 100 healthy controls, which have recently been published (14).

MATERIALS AND METHODS

The nature of the study was explained to, and informed consent obtained from, ten patients with RP from Western New York State. These patients represented seven unrelated families, four with autosomal dominant RP, two simplex presentations (no known family history) and one multiplex familiity with two of two affected siblings without prior family history. A diagnosis of RP was based on clinical presentation, family history and at least one family member with electroretinographic documentation of RP.

After an overnight fast of at least 12 h, plasma was obtained in citrated vacutainer vials by venipuncture for analysis of FA composition. Samples were centrifuged and the plasma transferred to freezing vials for storage at −20°C until analysis was performed at The Hormel Institute (Austin, MN).

Total lipids were extracted from plasma with chloroform/methanol (2:1, vol/vol), the water layer removed, the extract filtered to remove protein and the chloroform layer blown to dryness. The residual lipids were dissolved in a minimum amount of chloroform and applied to a silicic acid thin-layer chromatography plate and developed in a mixture of petroleum ether (b.p. 30-60°C), diethyl ether and acetic acid (80:20:1, by vol) to separate the PL, NEFA, TG and CE. Each lipid fraction was scraped separately from the plate, extracted from silicic acid, esterified with 12% BF_3 in methanol to form the fatty acid methyl esters (FAME), and then extracted with petroleum ether (b.p. 30-60°C) for capillary gas chromatographic analysis.

A model 428 chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 50 m × 0.25 mm bonded 007 FFAP-based silica capillary column (Quandrex, New Haven, CT) was used to separate the FAME. The instrument temperature was programmed from 170 to 220°C at 2°C/min with a final hold of 25 min, separating 12:0 to 22:6ω3. The detector temperature was 250°C. Helium

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Abbreviations: CE, cholesteryl ester; DBI, double bond index; DHA, docosahexaenoic acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; FA, fatty acids; FAME, fatty acid methyl esters; LCAT, lecithin-cholesterol acyl transferase; MMP, mean melting point; NEFA, nonesterified fatty acids; PL, phospholipids; PUFA, polyunsaturated fatty acids; ROS, rod outer segments; RP, *Retinitis pigmentosa;* RPE, retinal pigment epithelium; TG, triglycerides.

carrier gas was used at a flow rate of 1.4 mL/min and a split ratio of 1:65. The FAME were identified by comparison with authentic FAME standards, and peak areas were integrated as wt% with a microprocessor. Individual peaks were distinguished representing as little as 0.05% of the FAME in the fraction.

Statistical analysis of the data was performed with the PC-SAS package (15) on a microcomputer. Significance testing for differences between the RP and control groups was performed using Student's *t*-test. For our primary hypothesis (abnormal DHA levels in RP patients), the resulting *P* value should be taken at face value. For all other results (25 measured variables), single differences at the $P < 0.05$ level should be interpreted cautiously as preliminary findings because interdependent variables are expected to show such a correlation by chance alone. Differences with $P < 0.01$ level are more persuasive, and those variables showing $P < 0.001$ remain highly significant even taking into account the numerous comparisons. Significant patterns of change in metabolically related fatty acids cannot be explained by chance.

The normalcy ratio is calculated by dividing the average of the RP group by the average of the controls, and *P* values are calculated on the differences between the two groups. Normalcy ratios are the equivalent of Z-scores.

Mean melting point (MMP) is a measure of lipid fluidity based upon the measurement of the full range of FA in a lipid class. It is based upon the near linearity of the plot of mixed melting points *vs.* composition of mixtures. It is the sum of the products of mole fraction × melting point for each FA in the sample:

$$MMP = \Sigma(MF_1)\,(MP_1) + (MF_2)\,(MP_2) \cdots (MP_n)\,(MP_n)$$

where MF is the mole fraction and MP is the melting point. MMP has the advantage that it measures an inherent property of each of the FA present, and does not involve the use of external probes unnatural to lipids.

Double bond index (DBI) is the number of double bonds per acyl group within a lipid, and heretofore has been a useful indicator of fluidity, based upon the content of unsaturated FA in the lipid. For example, DBI of PL of normal rats is above 1.5, whereas severely essential fatty acid (EFA)-deficient rats exhibit values as low as 0.8 (16). MMP improves upon DBI by including contributions to fluidity by saturated and branched FA of the full range of chain lengths.

RESULTS

Plasma PL. Compositions, reflecting PL composition of tissue membranes, are given in Table 1.

Analysis of the PL from RP patients showed that the nutritionally EFA, α-linolenic (18:3ω3) and linoleic (18:2ω6), are present in normal proportions. However, the polyunsaturated long-chain FA 20:4ω6, 22:4ω6 and 22:5ω6 of the ω6 series, as well as 22:5ω3 and 22:6ω3 of the ω3 family, are significantly lower than in the healthy controls. Total saturated FA are found in higher proportion in the RP patients, and the individual components accounting for this rise are shown in Table 1 and in the graphic profile for PL in RP shown in Figure 1. Total monounsaturated FA are not abnormal in the PL. The total PUFA and DBI are greatly diminished as shown by the high level of statistical significance when compared to the 100 healthy controls. The products of Δ5 and Δ4 desaturation, and the products of elongation of C_{20} to C_{22} FA, shown in Figure 1, are diminished in PL in the RP group.

TABLE 1

Fatty Acids of Plasma Phospholipids in *Retinitis pigmentosa* (RP) and Normal Controls

Fatty acid	RP (n = 10)	SEM	Controls (n = 100)	SEM	Probability *(P)*
18:2ω6	22.49	1.24	23.90	0.28	
18:3ω6	0.14	0.02	0.12	0.01	
20:2ω6	0.41	0.05	0.47	0.02	
20:3ω6	3.48	0.32	3.41	0.08	
20:4ω6	10.46	0.55	12.81	0.19	<0.001
22:4ω6	0.42	0.02	0.76	0.03	<0.01
22:5ω6	0.39	0.02	0.60	0.02	<0.01
18:3ω3	0.16	0.02	0.21	0.01	
20:5ω3	0.55	0.06	0.59	0.03	
22:5ω3	0.72	0.03	1.13	0.03	<0.001
22:6ω3	2.36	0.13	3.59	0.11	<0.01
20:3ω9	0.13	0.03	0.13	0.01	
14:0	0.32	0.03	0.20	0.01	<0.01
16:0	26.69	0.72	21.05	0.20	<0.001
18:0	13.81	0.31	12.53	0.16	<0.05
20:0	0.26	0.02	0.33	0.02	
22:0	0.69	0.02	1.11	0.04	<0.001
24:0	0.46	0.02	0.88	0.07	
16:1ω7	0.90	0.06	0.70	0.02	<0.01
18:1ω9	9.98	0.43	8.87	0.16	<0.05
20:1ω9	0.13	0.02	0.22	0.02	
22:1	0.06	0.01	0.01	0.00	
24:1	0.69	0.08	1.20	0.04	<0.01
Branched	1.18	0.11	1.05	0.05	
Odd-chain	0.21	0.02	0.19	0.01	

Plasma CE. The FA compositions of CE are given in Table 2, and the full FA profile is shown in Figure 2. Total ω3 and all individual ω3 FA were normal with the exception of 20:5ω3 (eicosapentaenoic acid, EPA) that was depressed. Similarly, the ω6 series showed only a single

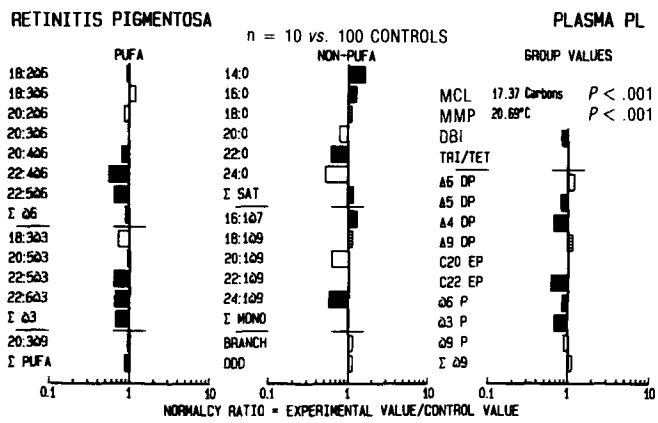

FIG. 1. Fatty acid profile of plasma phospholipids (PL) in ten patients with *Retinitis pigmentosa*. MCL is the mean chain length in carbon atoms, MMP is the calculated mean melting point of the inherent fatty acids and DBI is the double bond index. Abbreviations: PUFA, polyunsaturated fatty acids; SAT, saturated; EP, elongation products; DP, desaturation products; p, probability; TRI/TET, triene/tetraene ratio, 20:3ω9/20:4ω6.

TABLE 2

Fatty Acids of Plasma Cholesteryl Esters in *Retinitis pigmentosa* (RP) and Normal Controls

Fatty acid	RP (n = 10)	SEM	Controls (n = 99)	SEM	Probability *(P)*
18:2ω6	53.78	1.80	56.59	0.42	
18:3ω6	1.21	0.12	0.90	0.04	<0.05
20:2ω6	0.10	0.02	0.05	0.02	
20:3ω6	0.90	0.09	0.87	0.04	
20:4ω6	7.06	0.91	7.84	0.18	
22:4ω6	0.02	0.01	0.01	0.00	
22:5ω6	0.13	0.06	0.02	0.00	<0.001
18:3ω3	0.44	0.04	0.43	0.02	
20:5ω3	0.17	0.05	0.51	0.03	<0.01
22:5ω3	0.01	0.01	0.02	0.00	
22:6ω3	0.42	0.03	0.47	0.03	
20:3ω9	0.07	0.01	0.00	0.00	
14:0	0.52	0.05	0.66	0.03	
16:0	9.77	0.35	9.76	0.14	
18:0	1.02	0.11	9.96	0.03	
20:0	0.11	0.03	0.02	0.01	<0.05
22:0	0.39	0.12	0.00	0.00	<0.001
24:0	0.05	0.02	0.00	0.00	
16:1ω7	3.13	0.43	2.64	0.08	
18:1ω9	15.21	1.01	15.62	0.18	
20:1ω9	0.09	0.01	0.00	0.00	
24:1	0.02	0.02	0.00	0.00	
Branched	2.12	0.42	0.74	0.05	<0.001
Odd-chain	0.20	0.02	0.20	0.01	

TABLE 3

Fatty Acids of Plasma Triglycerides in *Retinitis pigmentosa* (RP) and Normal Controls

Fatty acid	RP (n = 10)	SEM	Controls (n = 101)	SEM	Probability *(P)*
18:2ω6	21.43	1.98	21.02	0.44	
18:3ω6	0.67	0.07	0.51	0.02	
20:2ω6	0.24	0.03	0.25	0.02	
20:3ω6	0.40	0.02	0.43	0.02	
20:4ω6	1.46	0.10	1.64	0.06	
22:4ω6	0.22	0.02	0.22	0.02	
22:5ω6	0.14	0.01	0.19	0.02	
18:3ω3	1.08	0.19	0.93	0.04	
20:5ω3	0.17	0.04	0.15	0.01	
22:5ω3	0.29	0.02	0.34	0.02	
22:6ω3	0.38	0.05	0.37	0.03	
20:3ω9	0.21	0.03	0.14	0.01	
14:0	1.89	0.24	1.48	0.08	
16:0	22.28	1.24	21.75	0.34	
18:0	2.75	0.23	3.61	0.11	<0.05
20:0	0.09	0.01	0.03	0.01	<0.05
22:0	0.06	0.03	0.04	0.01	
16:1ω7	4.98	0.40	4.14	0.11	<0.05
18:1ω9	36.97	1.36	37.83	0.37	
20:1ω9	0.33	0.03	0.04	0.01	<0.001
22:1	0.02	0.01	0.00	0.00	
Branched	1.25	0.10	1.06	0.06	
Odd-chain	0.24	0.02	0.32	0.01	

abnormal value, namely 22:5ω6, which was significantly elevated. Several saturated FA were elevated, as were ω9 FA. Summary indices showed small diminutions of total PUFA and products of Δ6 desaturation. Other product/substrate ratios were normal.

Plasma TG. TG compositions for the RP patients and controls are given in Table 3, and the full graphic profile for plasma TG, including the ω6 and ω3 elongation and desaturation products, is shown in Figure 3. The total saturated FA were different from the control group. Although the total of monounsaturated FA were normal, 20:1ω9 was significantly elevated. Total PUFA and the DBI were normal as were the products of desaturation and elongation.

Plasma NEFA. NEFA plasma levels are given in Table 4, and a full graphic profile of NEFA is shown in Figure 4. The sole ω3 FA to show an abnormality was 22:5ω3, which was significantly elevated in RP patients. Total ω6 FA were depressed in the NEFA fraction, as were 18:2ω6 (linoleic acid) and 20:4ω6 (arachidonic acid), the dominant individual ω6 FA. However, the products of the elongation and desaturation of 20:4ω6, 22:4ω6 and 22:5ω6 were significantly elevated. Nearly all saturated FA were elevated, as were Δ9 desaturation and elongation products.

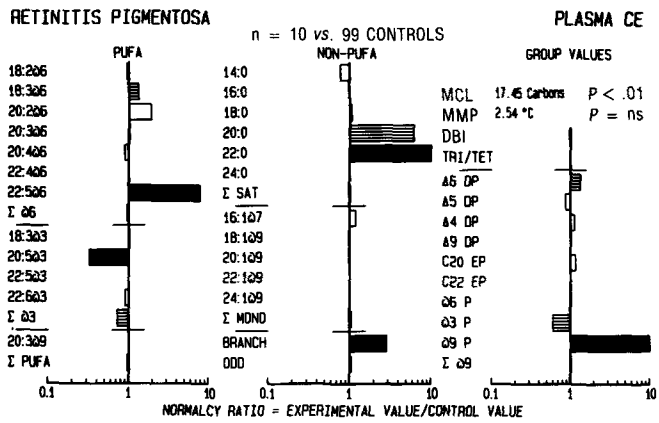

FIG. 2. Fatty acid profile of plasma cholesteryl esters (CE) in ten patients with *Retinitis pigmentosa*. See Figure 1 for abbreviations; ns, not significant.

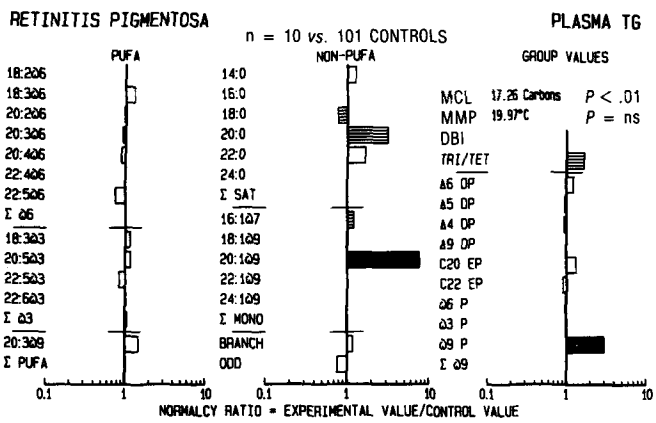

FIG. 3. Fatty acid profile of plasma triglycerides (TG) in ten patients with *Retinitis pigmentosa*. See Figures 1 and 2 for abbreviations.

TABLE 4

Fatty Acids of Plasma Nonesterified Fatty Acids in *Retinitis pigmentosa* (RP) and Normal Controls

Fatty acid	RP (n = 10)	SEM	Controls (n = 98)	SEM	Probability *(P)*
18:2ω6	14.22	1.13	18.39	0.28	<0.001
18:3ω6	0.27	0.03	0.15	0.03	
20:2ω6	0.25	0.02	0.15	0.02	
20:3ω6	0.47	0.08	0.52	0.05	
20:4ω6	0.78	0.05	2.42	0.13	<0.001
22:4ω6	0.27	0.04	0.06	0.01	
22:5ω6	0.68	0.22	0.05	0.01	<0.001
18:3ω3	0.81	0.08	1.08	0.05	
20:5ω3	0.12	0.04	0.05	0.01	
22:5ω3	0.59	0.22	0.10	0.02	<0.001
22:6ω3	0.41	0.11	0.32	0.06	
20:3ω9	0.07	0.02	0.01	0.00	
14:0	1.77	0.13	1.14	0.06	<0.01
16:0	23.30	0.97	19.37	0.27	<0.001
18:0	11.48	0.96	12.10	0.31	
20:0	0.32	0.04	0.02	0.01	<0.001
22:0	1.29	0.21	0.06	0.02	<0.001
24:0	0.31	0.15	0.00	0.00	<0.001
16:1ω7	3.17	0.50	2.86	0.10	
18:1ω9	32.10	1.09	37.34	0.52	<0.01
20:1ω9	0.32	0.02	0.01	0.00	<0.001
24:1	0.20	0.04	0.01	0.00	<0.001
Branched	1.70	0.15	0.83	0.07	<0.001
Odd-chain	0.37	0.03	0.25	0.02	

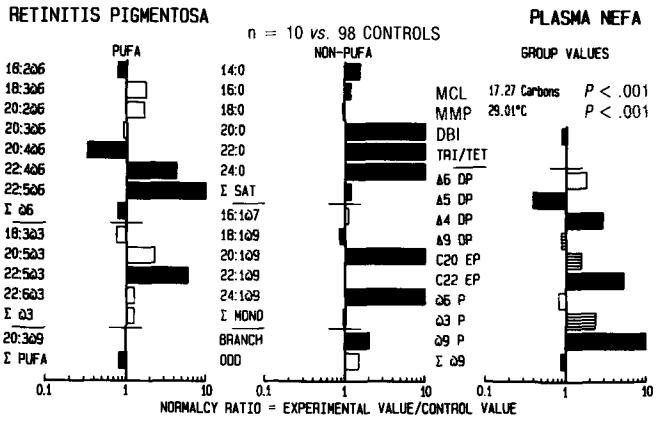

FIG. 4. Fatty acid profile of plasma nonesterified fatty acids (NEFA) in ten patients with *Retinitis pigmentosa*. See Figure 1 for abbreviations.

DISCUSSION

PL. The normal levels of the dietary precursor 18-carbon EFA in the PL fraction of RP patients suggest normal dietary intake and intestinal absorption of these nutrients. This observation is also supported by normal EFA levels in the TG fraction, a reflection of recent dietary intake.

The products of α-linolenic acid, 18:3ω3, in PL after undergoing Δ6 desaturation, elongation to C_{20}, and Δ5 desaturation remain at normal levels in RP. However, the C_{22} chain elongation product, 22:5ω3 (DHA) is significantly low in the RP group as is 22:6ω3 (DHA), the Δ4 product. For the ω6 series, the Δ6 desaturation and C_{20} chain elongation products appear to be normal in the RP group, but Δ5 desaturation, C_{22} elongation and final products all are lower than normal. These blocked steps are illustrated in Figure 1 by arrows. In the ω3 series, the blockage is at elongation to C_{22}, whereas in the ω6 series it is at Δ5 desaturation.

Δ6 and Δ5 desaturation and chain elongation steps to C_{20} and C_{22} acids occur in many tissues, but the liver may be the most important site physiologically (17). The final Δ4 product, derived *via* retroconversion (18), has been demonstrated in only a limited number of tissues, including human retinoblastoma cells (19,20), rat retina (21), rat adrenocortical cells (22), human fetal fibroblasts (20) and liver (17).

The highly significant depression of long-chain PUFA of plasma in the RP patients that we have studied suggests altered enzyme activity in tissues producing 22:5ω3, 22:6ω3, 20:4ω6 and 22:5ω6, notably liver. In this study the elongation of C_{20} PUFA appears to be the more affected step, leading to lesser levels of 22:5ω3 and 22:6ω3. If the ROS membranes of patients with RP have similarly reduced total PUFA and DBI, the consequent substantially reduced membrane fluidity, indicated by elevated calculated MMP, could contribute to biochemical alterations in visual pigment cycling, causing toxic injury to the outer retina and RPE.

Alternatively, the abnormal PL FA composition could represent a consequence of retinal injury rather than a cause. If we postulate a cause of the pathologic and pigmentary changes in RP unrelated to PUFA metabolism, breakdown of the blood retinal barrier would be a likely consequence. However, loss of PUFA from retinal tissue may tend to elevate the blood PL PUFA levels, at least temporarily. Thus, if systemic lipid changes are secondary in RP, a more complex mechanism than diffusion across a damaged blood retinal barrier must be at work.

CE analyses showed depression only of 20:5ω3 (EPA) in the ω3 series, which was modestly significant, and elevation of 22:5ω6 of the ω6 FA, which was highly significant. Lower content of the product of retroconversion of 22:6ω3 and higher content of substrate for ω6 retroconversion could explain these observations. The synthesis of plasma CE occurs largely in high density lipoprotein by action of lecithin-cholesterol acyl transferase (LCAT), as well as in liver. The changes observed in plasma PL are not reflected in plasma CE, although LCAT transfers the unsaturated acids from position 2 of phosphatidylcholine to cholesterol.

TG results, given in Table 2, showed completely normal proportions of all PUFA, suggesting normal dietary intake and adsorption as mentioned above. Saturated and monounsaturated totals were also normal, but several individual saturated FA were elevated or depressed, and Meade's FA (20:3ω9) was very high despite normal levels of EFA. The 20:3ω9 is the product of Δ5 desaturation in the ω9 series of which oleic acid is the precursor. Although 20:3ω9 was not elevated in plasma PL, its increase in TG is an indication that Δ5 desaturation may have been elevated in RP, and that the nonfunctional product, 20:3ω9, may have been shunted into TG.

NEFA demonstrated normal total ω3 FA, and normal individual FA with the exception of elevated 22:5ω3. Depressed 18:2ω6 in NEFA with normal PL and TG levels

suggests that linoleic acid may have been shunted away from adipose tissue. NEFA are the preferred energy source for some metabolically active tissues, such as heart, in transit from the adipose tissue and other storage sites. Recently, retroconversion of C_{22} and C_{20} PUFA in fibroblasts and vascular endothelial cells has been documented (17). Diminished retroconversion of C_{22} to C_{20} PUFA could explain the apparent increase of C_{22} elongase activity in our NEFA results.

FA profiles of plasma lipids provide rapid visual comparison of individual FA or groups of FA in two populations, and the changes of profile shown here may be compared readily with similar previous comprehensive studies of other diseases, nutritional states and physiological states (23-25). By expressing the compositions of FA as ratios to normal values on a logarithmic scale, bars for equifold increases or decreases appear as equal changes in concentrations of substrate FA, affecting metabolic kinetics equally. The profiles measured upon plasma or serum PL are a measure of those in structural membranes in body tissues, because the same deficiencies of PUFA measured analytically occur in PL of tissues as occur in plasma PL of nutritionally EFA-deficient animals and man. FA profiles assess the individual or group status with respect to both ω6 and ω3 EFA, and also indicate metabolic aberrations in the metabolism of PUFA if they occur. In instances of EFA deficiencies, the FA profile also indicates which nutritionally nonessential FA increase in replacement of the deficient EFA. For example, in Figure 1 the very significant decreases of 20:4ω6, 22:4ω6, 22:5ω6, 22:5ω3 and 22:6ω3 are compensated by increases of saturated and monounsaturated FA with less than 20 carbon atoms, whereas saturated and monounsaturated FA with more than 18 carbon atoms are suppressed, all in a stepwise fashion. Similar observations led to the calculation of estimated MMP as a measure of fluidity (22-24).

In the present study, DBI of plasma PL was consistently lower in the ten RP patients than the DBI of controls, averaging 83.9% of that value. MMP of the plasma PL of RP patients was consistently higher than the MMP of the control group, averaging 140% of control mean. Thus, the relative difference from control value was more than twice as great for MMP as for DBI, making MMP the more discriminating index of fluidity.

Relationship to protein defects in RP. Recently, amino acid substitutions in the visual pigment rhodopsin have been found in 25-30% of RP families, this protein defect being found only in the affected individuals in the pedigree (26,27). The mechanism of retinal injury in these patients remains unknown, and it is also not known whether rhodopsin defects are associated with abnormal long-chain PUFA. Further investigation will be necessary to determine whether abnormal PUFA and rhodopsin may define biochemically distinct RP groups.

The data presented here indicate that patients with RP have significantly low levels of both ω6 and ω3 PUFA. Deficiency of ω3 FA has been demonstrated to be associated with neuropathy (28). The retina is an extension of the nervous system, and abnormalities of vision observed in RP are demonstrated here to be associated with long-chain ω3 PUFA depletion of FA of circulating PL. Although dietary supplies of 18:3ω3 seemed to be normal in the RP patients, their long chain ω3 PUFA and ω6 PUFA were deficient. These long-chain FA occur in fish and meat products and might be enhanced in plasma PL by supplementation with fish oil (25) or by diets containing fish and meat (29). Measurement of plasma response in RP patients to dietary supplementation may be an appropriate first step to determine suitable intake levels of ω3 and ω6 long-chain PUFA prior to comparative clinical studies.

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Effects of High Corn Oil Diet on Preneoplastic Murine Colons: Prostanoid Production and Lipid Composition

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In the present study, the effect of normal (5% by wt) and high (23.5% by wt) corn oil diets on prostanoid production and on the lipid composition of preneoplastic colonic epithelium was investigated. CF_1 mice (female, 3–4-weeks-old) were fed a normal corn oil diet *ad libitum* and were treated with the colon carcinogen 1,2-dimethylhydrazine (DMH, 20 mg/kg/wk) or saline (control) for 24 wk. At this stage, all animals received the AIN-76 diet (normal corn oil) *ad libitum*. Following the last injection, half of the animals from each treatment group were randomly allocated to a high corn oil diet for 5 to 10 wk, whereas the remaining animals continued on the normal corn oil diet. After 5 wk of feeding, the colonic mucosa of carcinogen-treated animals had a higher level of bicyclic prostaglandin E_2 (PGE_2) than had the animals in the control groups; prostanoid synthesis in the colonic mucosa of control animals was unaffected by the high corn oil diet. Preneoplastic colonic mucosa of animals fed the high corn oil diet had a significantly higher level of PGE_2 than corresponding control colonic mucosa. The 6-keto-prostaglandin $F_{1\alpha}$/thromboxane B_2 ratio was significantly lower in the DMH-treated groups than in the control groups, and was unaffected by dietary treatments. After 10 wk of feeding a particular diet, the differences in the fatty acid composition between the control and DMH-treated groups were minor. Our findings demonstrate that the preneoplastic colonic epithelium differs from that of normal epithelium with respect to prostanoid synthesis.
Lipids 29, 67–71 (1994).

As dietary factors are recognized as being important in modulating the carcinogenic process, dietary fats have received particular attention. Epidemiological studies suggest that there is an association between the consumption of a high-fat diet and the incidence of colon cancer (1,2), which is supported by studies on animal models (3). The effect of dietary fats on colon tumorigenesis depends not only on the amount of fat consumed, but also on the type of fat and its fatty acid composition (3).

Prostanoids derived from arachidonic appear to be important in the tumorigenic process (4–9), and neoplastic cells have been shown to produce more prostaglandins than do normal surrounding tissue. It has also been shown that inhibitors of prostaglandin synthesis inhibit colon tumorigenesis and aid regression of adenomatous polyps in patients with familial polyposis (10–12). One mechanism by which dietary fats may affect colon tumorigenesis is by modulating eicosanoid synthesis in the colonic mucosa (13).

In a previous study (14), we demonstrated that normal colonic epithelia (without preneoplastic changes) of animals fed high corn oil or beef tallow diets had higher levels of phosphatidylcholine compared to those of animals on corresponding low-fat diets. The fatty acid composition of the total phospholipids and triacylglycerols reflected the composition of the dietary fat. It was noteworthy that the levels of three eicosanoids, prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), which are well known to be involved in the carcinogenic process (4–6), were unaffected by the dietary fat regimens.

Since preneoplastic or neoplastic cells may respond differently to environmental factors than do normal cells, we reasoned that the preneoplastic colonic tissue may respond differently to dietary lipids than would its normal counterpart.

The main objective of the present study was to assess the effect of a high corn oil diet on the lipid composition and the production of PGE_2, PGI_2 and TXA_2 in preneoplastic colonic tissue.

METHODS AND MATERIALS

Animals, diets and carcinogen treatment. Three- to four-week-old female CF_1 mice were obtained from Charles River Canada (Montreal, Quebec, Canada). The mice were housed five to a plastic cage in a room under controlled environmental conditions (21°C) with a 12-h light–dark cycle. After one week of acclimatization, the mice were randomly divided into two groups. One group was given weekly intraperitoneal injections of 1,2-dimethylhydrazine (DMH) (Aldrich, Milwaukee, WI) at a dose of 20 mg/kg body weight for 24 wk. The DMH was dissolved in 1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.5. The other group was injected with vehicle and served as control. The mice were fed an AIN-76 diet (15), which contained 5% corn oil by weight, for up to 24 wk. At various time intervals, including 16 and 24 wk after initiation of DMH treatment, three animals from the carcinogen-treated and the control groups were killed and their colons examined histologically to monitor neoplastic changes. At week 24, the animals were randomly allocated to either a normal or a high corn oil diet (20 DMH-treated and 20 control animals/diet). The high corn oil diet contained 23.5% corn oil by weight, and was made by substituting corn oil for an isocaloric amount of sucrose (Table 1). Animals were killed at 5 wk to determine prostanoid production, and at 10 wk to determine mucosal fatty acid composition.

Determination of prostanoids. Five DMH-treated mice and five control mice fed either the high or the normal corn oil diet were killed by cervical dislocation. The colons were removed and rinsed thoroughly in Krebs Ringer, the mucosae were separated by scraping with a glass slide and the scrapings were placed in tubes containing 20 mL of Krebs Ringer. Tissue weights were determined, and the tissues were incubated for 30 min at 37°C in Krebs Ringer to allow for synthesis of prostanoids from endogenous sources as described previously (14). The amount of prostanoids produced by the mucosal tissue was determined by radioimmunoassay (Amersham Corp., Arlington

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Abbreviations: DMH, 1,2-dimethylhydrazine; EDTA, ethylenediaminetetraacetic acid; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$.

TABLE 1

Percent Composition of Diets (%, w/w)

Ingredients	High corn oil	Normal corn oil
Casein	23.5	20.0
DL-methionine	0.4	0.3
Dextrin	17.6	15.0
Solka floc	5.9	5.0
Sucrose	23.5	50.0
Corn oil	23.5	5.0
AIN mineral mix	4.1	3.5
AIN vitamin mix	1.2	1.0
Choline bitartrate	0.2	0.2

Heights, IL). In the case of PGE_2, the assay system is based on the conversion of the major PGE_2 metabolite 13,14-dihydro-15-keto-prostaglandin E_2 to the stable, bicyclic compound 11-deoxy-13,14-dihydro-15-keto-11,16-cyclo-prostaglandin E_2 (bicyclic PGE_2). This assay utilizes antiserum specific for bicyclic PGE_2. The cross-reactivity of this radioimmunoassay with prostaglandin E_1 is less than 0.01%, and with TXB_2 or $PGF_{1\alpha}$ is less than 0.0001%. The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ assay was 5.1% for PGE_2 and 0.014% for TXB_2. Cross-reactivity of PGE_2 and $PGF_{1\alpha}$ with the TXB_2 antiserum was 0.003% or less. TXA_2 and PGI_2 are both very unstable compounds and readily undergo hydrolysis to form the stable inactive metabolites thromboxane B_2 (TXB_2) and 6-keto-prostaglandin in $F_{1\alpha}$ ($PGF_{1\alpha}$).

Determination of colonic fatty acids. Mice from the control and DMH-treated groups were fed the normal or high corn oil diets for 10 wk, after which time their colons were removed, washed thoroughly in Krebs Ringer, and the mucosal and muscle layers were separated. Ten animals from each treatment were used, and two mucosae were pooled for each analysis.

The tissues were placed immediately in chloroform/methanol (2:1, vol/vol), and lipids were extracted as described by Folch *et al.* (16). The major lipid classes were separated by thin-layer chromatography, and fatty acid methyl esters were prepared by the method of Morrison and Smith (17). The fatty acid methyl esters were analyzed by gas chromatography using a Hewlett Packard 5890A instrument equipped with a flame-ionization detector (Palo Alto, CA) and with a fused silica capillary column, 30 m × 0.25 mm. The gas chromatograph was programmed from 190 to 220°C at 10°C/min using helium as carrier gas at a flow rate of 45 cm/s.

Statistical analysis. Statistical significance of the data was determined by analysis of variance in combination with Duncan's Multiple Range Test at $P \leq 0.05$.

RESULTS

In the present study the test animals were given weekly injections of 1,2-DMH for 24 wk. None of the animals developed polyps within 24 wk, but showed microscopic lesions when examined histologically (Fig. 1). Histological sections of the colons treated with carcinogen revealed the presence of preneoplastic changes in the crypts characterized by mild to severe dysplasia (18). At week 34, two out of ten animals in the normal and six out of ten animals in the high corn oil group had macroscopic lesions.

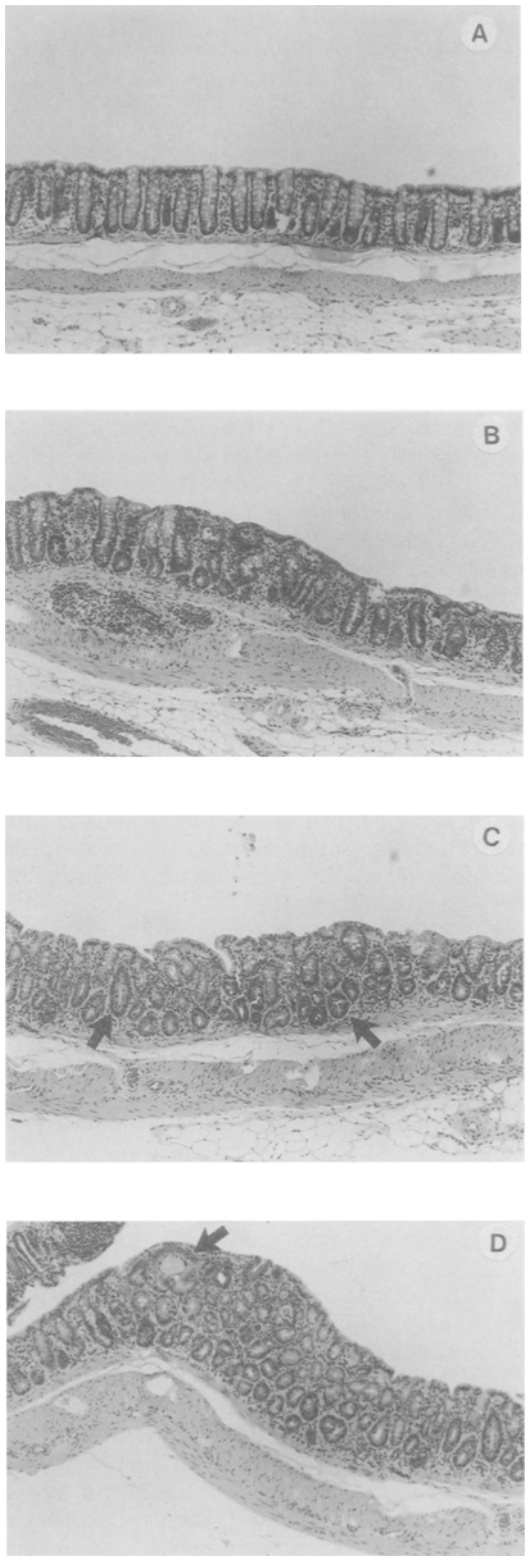

FIG. 1. Histological sections of mouse colons (H & E × 400). Mice were treated with vehicle solution (A) or DMH for 24 wk (B, C, D). A. Normal colon depicting longitudinal section of crypts. B–D. Preneoplastic epithelium exhibiting histological and proliferative atypia. B. Absence of complete longitudinal sections of crypts because of increased tortuosity. C. Note the presence of dysplasia, the absence of goblet cells (arrow), and the increased mitosis (arrow). D. In addition to changes described in B and C, note the presence of a prominent focal preneoplastic lesion (arrow).

TABLE 2

Prostanoid Synthesis in Control and DMH-Treated Colonic Mucosa of CF_1 Mice Fed Normal and High Corn Oil Diets[a]

Prostanoid measured	Normal corn oil		High corn oil	
	Control	DMH-treated	Control	DMH-treated
Bicyclic PGE_2	2.47 ± 0.32[b]	3.06 ± 0.84[b,c] (124)	2.43 ± 0.37[b]	4.31 ± 1.06[c] (178)
$PGF_{1\alpha}$	5.82 ± 2.1[b]	5.00 ± 1.87[b] (85.8)	5.66 ± 2.12[b]	3.54 ± 1.11[b] (52.4)
TxB_2	0.33 ± 0.04[b]	0.66 ± 0.11[d] (202.7)	0.28 ± 0.04[b]	0.46 ± 0.02[c] (167)
$PGF_{1\alpha}/TxB_2$ ratio	16.8 ± 4.0[c]	6.7 ± 1.7[b]	18.6 ± 4.6[c]	8.1 ± 1.2[b]

[a]Mice were injected with DMH for 24 wk then allowed to eat a normal or a high corn oil diet for 5 wk. Values given are the mean (n = 5) of five samples of colonic tissue ± SEM expressed as ng/g/30 min. Means in a row without a common superscript [b,c,d] are significantly different ($P < 0.05$). Numbers in parentheses represent values as percent of control. Abbreviations: DMH, 1,2-dimethylhydrazine; PGE_2, prostaglandin E_2; $PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; TxB_2, thromboxane B_2.

In control animals, synthesis of PGE_2, TXA_2 and PGI_2 in the colonic mucosae measured as their stable metabolites, *i.e.*, bicyclic PGE_2, TXB_2, and $PGF_{1\alpha}$, respectively, was unaffected by the dietary treatments at 5 wk (Table 2), consistent with our earlier observations (14). The colons treated with DMH had higher bicyclic PGE_2 levels than the control colons, but this effect was significant only for the high corn oil, DMH-treated group. The values for preneoplastic colons from the high or the normal corn oil groups were not significantly different from each other. The levels of $PGF_{1\alpha}$ were similar in all groups. The TXB_2 levels were significantly higher for the DMH-treated groups than the control groups. In this case, the normal corn oil DMH-treated group exhibited the highest value (202% of the control). The ratio between $PGF_{1\alpha}$ and TXB_2 was significantly lower for the DMH-treated groups compared to the controls, but the effect of the dietary treatment was not evident.

Changes in the fatty acid composition between control and DMH-treated mucosa for a particular dietary treatment were generally minor (Table 3). The phospholipid fractions of the high corn oil group had decreased levels of 18:1 with a concomitant increase in 18:2 and 20:3 compared to the normal corn oil group. The level of 20:4 in the mucosal phospholipids did not vary across the treatment groups. The only significant DMH-treatment effect seen was a small rise in 18:2. In addition, a significant ($P < 0.05$) decrease in the ratio of 18:0/18:1 in the phospholipid fractions of normal corn oil DMH-treated mucosa was noted when compared with the control mucosa, but this was not seen in the high corn oil group. The fatty acid composition of the mucosal triglycerides is shown in Table 4. Among the mucosal triglycerides, the normal corn oil DMH-treated group contained less 18:1 and more 18:2 than the control group. Similar changes in the lipid composition of colonic mucosa were seen as early as within 5 wk of dietary treatments (results not shown).

TABLE 3

Percent Fatty Acid Composition of Control and DMH-Treated Mucosal Phospholipids in Mice Fed Normal and High Corn Oil Diets[a]

Fatty acids	Normal corn oil		High corn oil	
	Control	DMH-treated	Control	DMH-treated
14:0	2.47 ± 0.32[b]	3.06 ± 0.84[b,c]	2.43 ± 0.37[b]	4.31 ± 1.06[c]
16:0	21.41 ± 0.85	22.76 ± 0.50	23.07 ± 0.82	22.65 ± 0.52
16:1	2.83 ± 0.23	1.89 ± 0.59	0.94 ± 0.30	1.53 ± 0.32
18:0	16.13 ± 0.29	14.92 ± 0.42	15.79 ± 0.51	16.31 ± 0.59
18:1	21.02 ± 0.43[c]	23.08 ± 1.45[c]	15.61 ± 0.60[b]	15.58 ± 0.41[b]
18:2	7.99 ± 0.32[b]	9.37 ± 0.95[c]	11.66 ± 0.50[d]	12.96 ± 0.29[e]
18:3	0.30 ± 0.18	0.50 ± 0.21	0.29 ± 0.18	ND
20:3	4.39 ± 0.32	4.66 ± 0.68	8.14 ± 0.29	6.86 ± 0.09
20:4	18.42 ± 0.54	17.56 ± 1.11	17.97 ± 0.43	17.36 ± 2.24
22:4	2.85 ± 0.22	2.08 ± 0.61	2.80 ± 0.98	2.54 ± 0.21
22:5	1.58 ± 0.21	1.74 ± 0.50	2.01 ± 0.57	1.88 ± 0.51
18:0/18:1	0.768 ± 0.019[c]	0.658 ± 0.064[b]	1.013 ± 0.019[d]	1.051 ± 0.054[d]
Total saturated	40.60 ± 0.61	39.15 ± 1.18	40.51 ± 1.20	41.29 ± 0.67
Total monounsaturated	23.85 ± 0.41[c]	24.97 ± 1.60[c]	16.55 ± 0.55[b]	17.11 ± 0.50[b]
Total polyunsaturated	35.52 ± 0.88	35.85 ± 1.55	42.86 ± 1.69	41.60 ± 0.40

[a]Values given are means ± SEM; n = 5 for each group and each n represents two pooled colonic mucosae. Lipid composition was determined after 10 wk of dietary treatments. Horizontal means without a common superscript [b–e] are statistically different ($P < 0.050$). Rows without any superscripts contain values that are not significantly different. ND, not detectable. See Table 2 for abbreviations.

TABLE 4

Fatty Acid Composition of Control and DMH-Treated Mucosal Triglycerides[a]

Fatty acids	Normal corn oil		High corn oil	
	Control	DMH-treated	Control	DMH-treated
14:0	1.15 ± 0.12	1.25 ± 1.00	0.43 ± 0.06	0.60 ± 0.06
16:0	18.41 ± 0.09^{c}	18.61 ± 1.36^{c}	10.15 ± 1.00^{b}	11.26 ± 0.44^{b}
16:1	7.59 ± 0.42^{c}	8.19 ± 0.66^{c}	3.20 ± 0.39^{b}	4.29 ± 0.45^{b}
18:0	1.25 ± 0.16^{c}	1.41 ± 0.18^{c}	0.68 ± 0.20^{c}	0.85 ± 0.15^{b}
18:1n-9	49.67 ± 0.99^{d}	44.08 ± 1.02^{c}	34.83 ± 1.02^{b}	34.21 ± 0.33^{b}
18:2n-6	20.91 ± 0.71^{b}	25.89 ± 1.78^{c}	49.56 ± 0.89^{d}	48.59 ± 0.43^{d}
18:3n-6	0.96 ± 0.07	0.56 ± 0.24	0.09 ± 0.05	0.21 ± 0.13
20:3n-6	ND	ND	0.05 ± 0.05	ND
20:4n-6	0.06 ± 0.06^{b}	ND	0.27 ± 0.11^{b}	ND
18:0/18:1	0.025 ± 0.003^{b}	0.030 ± 0.005^{b}	0.019 ± 0.006^{b}	0.025 ± 0.004^{b}
Total saturated	20.81 ± 1.22^{c}	21.27 ± 1.57^{c}	11.26 ± 1.23^{b}	12.71 ± 0.05^{b}
Total monounsaturated	57.27 ± 0.79^{d}	52.28 ± 0.71^{d}	38.77 ± 0.64^{b}	38.50 ± 0.28^{b}
Total polyunsaturated	21.92 ± 0.75^{b}	26.45 ± 1.63^{b}	49.97 ± 1.00^{c}	48.80 ± 0.38^{c}

[a]Values given are means ± SEM; n = 5, two pooled colonic mucosae/sample). Lipid composition was determined after 10 wk of dietary treatments. Means in a row without a common superscript $^{b-d}$ are significantly different ($P < 0.050$). ND, not detectable. See Table 2 for abbreviation.

DISCUSSION

Our objective was to investigate the effect of a high corn oil diet on prostanoid production and lipid composition of the preneoplastic colonic epithelium. Differences were observed between the colonic epithelium with preneoplastic changes and normal colon. The levels of PGE_2 in the DMH-treated, normal or high corn oil groups were 124 and 178% higher, respectively, than in the corresponding control groups. The ratio between the levels of $PGF_{1\alpha}$ and TXB_2, the stable metabolites of PGI_2 and TXA_2, was significantly lower in the DMH-treated groups than in the control groups. However, the fatty acid composition of the total phospholipid and triacylglycerol fractions was similar in the preneoplastic and normal colonic mucosa, regardless of the level of fat in the diet. The colonic mucosa of the high corn oil DMH-treated group synthesized substantially higher levels of PGE_2 than did normal epithelium. However, this effect was masked when the DMH-treated colonic epithelia from the normal corn oil group were included in the analyses. There was no difference in PGE_2 production between the two DMH-treated groups. Recently, Rao and Reddy (13) have reported that preneoplastic epithelium of animals fed a high corn oil diet synthesized significantly higher levels of PGE_2 than did normal epithelium; however, animals fed normal corn oil diets were not included in their analyses. The subtle increase in the synthesis of PGE_2 by preneoplastic colonic epithelium in the high corn oil group compared to the normal corn oil group may be significant when one considers the physiological potency of prostanoids.

It is somewhat perplexing that we found the PGE_2 levels in the preneoplastic murine colonic epithelium of animals on the normal corn oil diet in the DMH-treated group only moderately elevated when compared with controls. Yamaguchi *et al.* (19) reported significantly higher levels of PGE_2 in the colon of rats several weeks after they were exposed to a carcinogen. This may suggest that mice are more resistant to carcinogens or diet-induced changes in their colons than rats. Furthermore, the large intragroup variability observed in our study indicates that the number of animals per group should be larger. The ratio between the levels of $PGF_{1\alpha}$ and TXB_2 was significantly lower in the carcinogen-treated colonic mucosa, which supports the conjecture that an imbalance in the ratio of these two eicosanoids may be important in neoplastic cell growth (20). Since all eicosanoids measured in our study are derived from arachidonic acid, it can be suggested that the altered prostanoid production in preneoplastic colons is well-regulated and may reflect a metabolic and/or an adaptive response of the neoplastic cells.

It has been proposed that a low saturation index, reflected in a consistently lower ratio of stearic to oleic acid in total lipid extracts of tumor *vs.* nontumor areas, may be a marker for malignancy (21–23). Our findings partially support this suggestion. A significant decrease was observed in the ratio of 18:0/18:1 in the phospholipids of the mucosa in the normal corn oil DMH-treated group, but not in the high corn oil DMH-treated group. Although some of the changes were statistically significant, it is not clear to what extent the changes are biologically significant.

The changes in the production of prostanoids by preneoplastic colonic mucosa were not accompanied by changes in the fatty acid composition of total phospholipids or triglycerides. However, this does not exclude the possibility that changes could occur within different phospholipid classes. There is limited information on the factors that regulate the synthesis and utilization of prostanoids in tissues (24). Therefore, further work is required on the various phospholipid classes and activities of enzymes involved in prostanoid synthesis in the preneoplastic colonic mucosa and in preneoplastic lesions.

Our findings demonstrate that prostanoid production is modified in the preneoplastic colon and that colonic mucosa that is preneoplasticly altered exhibits marked changes in the ratio of $PGF_{1\alpha}$ and TXB_2. The significance of these changes and their relationships to dietary lipids and tumorigenesis require further evaluation.

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METHOD

An Improved Method for the Measurement of Malondialdehyde in Biological Samples

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An improved method was developed for measuring malondialdehyde (MDA) as its thiobarbituric acid (TBA) complex. Samples were initially incubated with 1% potassium iodide and 0.1% butylated hydroxytoluene at 50°C for 20 min, and then with 0.4% TBA at 60°C for 60 min. The MDA–TBA complex formed was extracted with isobutyl alcohol and measured by high-performance liquid chromatography with fluorescence detection. The improved method allows for a more specific determination of MDA present in biological samples.
Lipids 29, 73–75 (1994).

Due to its relative simplicity and high sensitivity, measurement of malondialdehyde (MDA) as thiobarbituric acid (TBA) adduct has been extensively used to assess the extent of lipid peroxidation in a variety of pathological conditions (1). In recent years, many improved procedures for measuring the MDA-TBA chromophore have been reported (1–3). Factors, such as reaction time, temperature and pH, as well as the presence of transition metals, antioxidants and reducing agents can markedly affect the formation of both the TBA–MDA chromophore and of artifacts during sample processing. We previously reported a selective and sensitive method for measuring MDA in biological samples using high-performance liquid chromatography (HPLC) with fluorescence detection (4). In the procedure described here, the possibility of artifact formation was further decreased by reacting the sample with KI prior to the formation of the MDA-TBA adduct. Thus, a more specific quantitation of MDA present in the assay system can be achieved. In addition, the quantitative relationship between conjugated dienes, hydroperoxides and MDA was examined in peroxidized fatty acids.

MATERIALS AND METHODS

Chemicals and reagents. Butylated hydroxytoluene (BHT), TBA, linoleic acid, linolenic acid, 1,1,3,3-tetramethoxypropane (TMP), KI, hydrogen peroxide (30%) and soybean lipoxygenase (type I) were purchased from Sigma Chemical Company (St. Louis, MO). Boric acid and isobutyl alcohol were from Mallinckrodt (St. Louis, MO); methanol (HPLC grade), glacial acetic acid and hydrochloric acid were from Fisher Scientific (Fair Lawn, NJ); tetrabutylammonium dihydrogen phosphate was from Aldrich Chemical Company (Milwaukee, WI); and ethanol (95%) was from Midwest Grain Products of Illinois (Pekin, IL).

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; LOH, hydroxy fatty acid; LOOH, fatty acid hydroperoxide; MDA, malondialdehyde; TBA, thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane.

Preparation of standards. The stock solution of MDA was prepared by dissolving 0.1 M TMP in 0.02 N HCl. Hydrogen peroxide, 0.1 μM, was prepared in distilled water. Working standards for MDA and hydrogen peroxide, ranging from 0.1–10.0 μM, were prepared fresh before use.

Enzymatic oxidation of fatty acids. Stock solutions of linoleic acid and linolenic acid (0.05 M) were prepared with 20% ethanol in 0.1 M potassium borate buffer, pH 8.0. The stock solution (0.5 μL) was mixed with 2.5 mL of 0.1 M borate buffer. Samples were oxidized by adding 3,000 units of lipoxygenase (5) at room temperature. The contents were then aliquoted before and 10 min after the addition of lipoxygenase for measuring the levels of lipid peroxidation products, MDA, lipid hydroperoxides and conjugated dienes.

Measurement of lipid peroxidation products. For MDA measurements, aliquots of the enzymatically peroxidized fatty acids (50–200 μL) and MDA standards (0.1–1.0 nmol) were added to test tubes that contained 0.1 mL of 0.1% BHT in 95% ethanol and 0.5 mL of 1% KI in methanol/acetic acid (2:1, vol/vol). The test tubes were then incubated at 50°C for 20 min. After adding 1 mL 0.4% TBA in 0.02N HCl, the contents were incubated at 60°C for 60 min, cooled to room temperature and extracted with isobutyl alcohol. The concentration of MDA was measured by HPLC with fluorescence detection using a C-18 reverse-phase column as described previously (4). Tetrabutylammonium dihydrogen phosphate, 0.5%, was added to the mobile phase (methanol/water, 1:1, vol/vol) as ion-pairing reagent.

The levels of diene conjugation in fatty acids were measured at 233 nm (6). Lipid hydroperoxides were quantified by the triiodide procedure described by Gebicki and Guille (7) using hydrogen peroxide as a standard.

Measurement of MDA in biological samples. To evaluate the applicability of the procedure to biological samples, freshly obtained human plasma was assayed for MDA levels. One-tenth to one-half mL of plasma was pipetted into a test tube that contained 0.5 mL of 1% KI in methanol/acetic acid (2:1, vol/vol) and 0.1 mL of 0.1% BHT in 95% ethanol. After incubation at 50°C for 20 min, the contents were mixed with 0.5 mL 0.4% TBA in 0.02N HCl and incubated at 60°C for 60 min. The samples were then extracted with 2 mL of isobutyl alcohol and measured for relative fluorescence by HPLC as described above. The differences between the sample means were analyzed using analysis of variance followed by Honest's multiple comparison test (Turkey Honest Significant Difference Test). A 95% ($P < 0.05$) of confidence level was used to determine statistical significance.

RESULTS

KI and BHT, individually or combined, had no effect on the formation of MDA–TBA chromophore over the

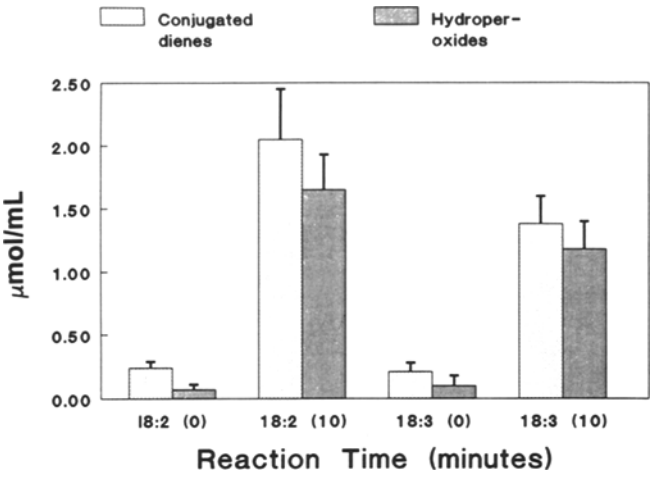

FIG. 1. Effect of KI and butylated hydroxytoluene on the formation of conjugated dienes and hydroperoxides. Linoleic acid (18:2) and linolenic acid (18:3) were incubated with lipoxygenase for 0 or 10 min. The reaction mixture consisted of 25 μmol fatty acid and 3,000 units of lipoxygenase in 3.0 mL of 0.1 M borate buffer (8.33 μmol fatty acid/mL). Results are expressed as μmol of conjugated dienes or hydroperoxides/mL of reaction mixture. The values represent means ± standard deviation of five experiments.

concentration range of 0.10–10 μM. The same was observed when the concentrations of KI and BHT were doubled or halved (results not shown).

The formation of conjugated dienes and hydroperoxides from linoleic acid and linolenic acid following reaction with lipoxygenase is shown in Figure 1. At 0 time, an average of 0.24 and 0.21 μmol/mL of conjugated dienes and 0.07 and 0.10 μmol/mL of hydroperoxides were detected for linoleic acid and linolenic acid, respectively. Ten minutes after the addition of lipoxygenase, an average of 2.05 and 1.38 μmol/mL of conjugated diene and 1.65 and 1.18 μmol/mL of hydroperoxides were found for linoleic acid and linolenic acid, respectively. This compares to less than 0.004 μmol/mL of MDA-TBA detected in the peroxidized

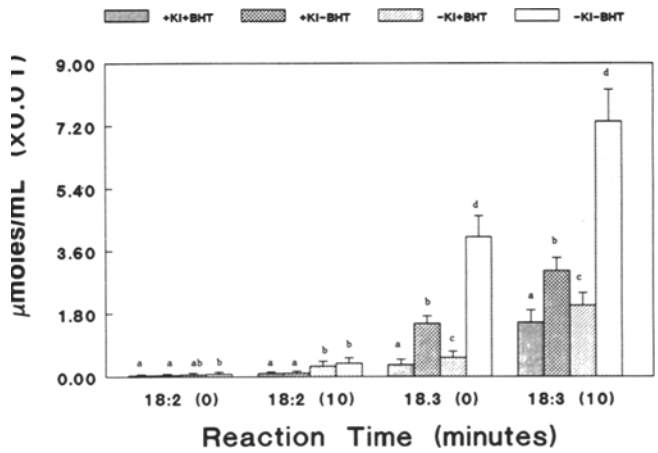

FIG. 2. Effect of KI and butylated hydroxytoluene (BHT) on the formation of malondialdehyde-thiobarbituric acid chromophore. Linoleic acid (18:2) and linolenic acid (18:3) were incubated with lipoxygenase for 0 or 10 min. The values represent the mean ± standard deviation of five experiments. See Figure 1 legends for additional experimental details. The means that have different lower case letters are significantly different ($P < 0.05$).

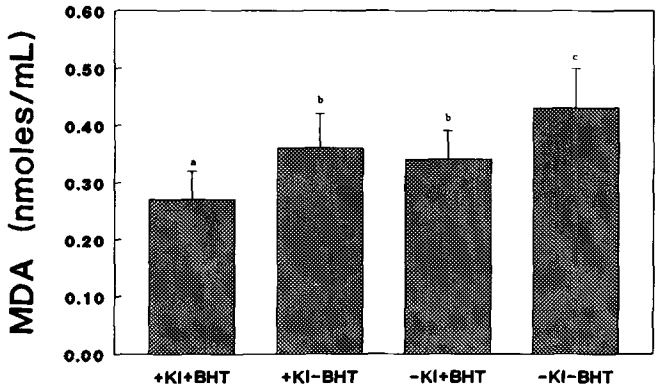

FIG. 3. Effect of KI and BHT on the measurement of MDA in human plasma. The values represent the mean ± standard deviation of fourteen samples. The means that have different lower case letters are significantly different ($P < 0.05$). See text for experimental details. Abbreviations: See Figure 2, and MDA, malondialdehyde.

linoleic acid and less than 0.08 μmol/mL in the peroxidized linolenic acid (Fig. 2) whether KI and/or BHT were included in the reaction mixture or not. As is also shown in Figure 2, the amounts of MDA-TBA found were highly variable depending upon the experimental conditions. The levels of MDA-TBA were decreased by the presence of BHT or KI in the reaction mixture; the addition of both KI and BHT had the greatest effect.

The applicability of the method to measuring MDA in biological samples was tested on human plasma samples. As is shown in Figure 3, the MDA values were significantly lower for plasma samples prepared with KI and/or BHT than for those without. The averaged values of 14 plasma samples were 0.27, 0.36, 0.34 and 0.43 nmol/mL for samples treated with +KI + BHT, +KI − BHT, −KI + BHLT and −KI − BHT, respectively.

DISCUSSION

Due to its sensitivity and simplicity, the measurement of the MDA-TBA chromophore has been widely employed to assess the extent of lipid peroxidation. However, autoxidation of unoxidized lipids and generation of MDA-like compounds during sample processing are among the most critical and confounding factors limiting the accurate measurement of MDA (1–4). In biological samples, the assay is further complicated by the nature of the peroxidized intermediates generated, their reactivity with other components, and by the presence of MDA-like compounds and their precursors. In the present study, an improved method for assaying MDA was developed based on procedures previously reported by us (4) and others (8–10). The key feature of the assay is the prevention or reduction of artifact formation by both KI and BHT during sample processing. A classic procedure for measuring lipid hydroperoxides in fats and oils is based on the ability of KI to reduce lipid hydroperoxides (LOOH), except endoperoxides, to their corresponding alcohols (LOH) (Reaction I) (8,9). Iodometric procedures have been preposed for measuring lipid hydroperoxides in biological samples (7,9).

$$LOOH + 2H^+ + 2\ I^- \rightarrow LOH + H_2O + I_2 \text{ (Reaction I)}$$

The ability of KI to reduce lipid hydroperoxides also reduces free radical generation *via* homolytic cleavage. In addition, KI and/or released I_2 may saturate double bonds of unsaturated fatty acids (10) and thus further decrease the autoxidation potential of the fatty acids present. KI seems to complement the antioxidant property of BHT. In the presence of both KI and BHT, the generation of MDA or MDA-like substances from unoxidized fatty acids, hydroperoxides or other biomolecules can thus be prevented or minimized. However, it remains possible that not all types of artifacts formation are prevented under these experimental conditions.

As is shown in Figures 1 and 2, only small and highly variable amounts of lipid hydroperoxides were converted to MDA. Therefore, estimation of MDA *per se* is not a reliable measure of lipid hydroperoxides. Similarly, as is also shown in Figure 1, the levels of conjugated dienes vary widely depending on the state of oxidation; thus, estimation of conjugated diene is not a suitable measure of lipid hydroperoxides, either.

It has long been recognized that MDA and other aldehydes can be metabolized *in vivo* and *in vitro* by aldehyde dehydrogenases and related enzymes (6,11-13). In addition, MDA may react with various cellular components and thereby decrease cellular levels. Thus, the MDA levels present in fresh biological samples can be expected to be very low. The MDA levels measured as TBA values and reported for normal human plasma varied widely and ranged from 0.32 to 47.2 nmol/mL (3). Whether the average MDA levels in human plasma measured in the present study, 0.27 nmol/mL, represents the steady-state concentration remains to be seen. Nevertheless, by further decreasing the possibility of artifact formation during sample processing, the present method is an improvement over the quite selective and sensitive method we reported previously (4), and affords a more specific measurement of MDA present in biological samples.

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COMMUNICATION

Molecular Species of Choline and Ethanolamine Glycerophospholipids in Rat Brain Myelin During Development

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The composition of the molecular species of various phospholipid subclasses was examined in myelin isolated from brain of 15-, 21- and 90-day-old rats. The molecular species of diacylglycerophosphocholine (PtdCho), diacylglycerophosphoethanolamine (PtdEtn) and plasmenylethanolamine (PlsEtn) were quantified by high-performance liquid chromatography (HPLC) after phospholipase C treatment and dinitrobenzoyl derivatization. In rat brain myelin, each phospholipid subclass showed a specific pattern of molecular species that changed during development. PtdCho contained large amounts of saturated/monounsaturated and disaturated species and low amounts of saturated/polyunsaturated species. During brain development, the levels of saturated/monounsaturated molecular species increased whereas those of the disaturated and saturated/polyunsaturated species decreased. PtdEtn were characterized by their low levels of disaturated species and a high content of saturated/monounsaturated and saturated/polyunsaturated species, of which those containing fatty acids of the n-3 series decreased, whereas those containing fatty acids of the n-6 series did not change during brain development. The levels of saturated/monounsaturated species increased in PtdEtn. No disaturated molecular species could be detected in PlsEtn. This alkenylacyl subclass contained large amounts of saturated/polyunsaturated, saturated/monounsaturated and dimonounsaturated molecular species. During development, the levels of saturated/polyunsaturated molecular species decreased while those of the two others increased. The data indicated that myelin sheaths undergo phospholipid changes during brain development and maturation.
Lipids 29, 77-81 (1994).

In the central nervous system, myelin is formed by oligodendroglial cells and constitutes the major portion of these cells (1,2). A number of observations support the idea that myelin sheaths are assembled by successive addition of myelin components to the plasma membrane of oligodendroglial cells, leading to changes in the composition of this membrane during maturation (3-6). During the early stage of myelin development, myelin lipids vary among species more than do the lipids of other subcellular fractions (7,8). Changes have also been observed for the various phospholipid classes. As myelination proceeds, the content of phosphatidylcholine (PtdCho) on a percent basis decreases, whereas plasmenylethanolamine (PlsEtn) and polyphosphoinositide levels increase several fold (3,8-10). Moreover, each class of phospholipids is characterized by a specific fatty acid composition suggesting that each class is a mixture of a large number of well-defined molecular species (4,11,12). The fact that the phospholipid fatty acid distribution changes during myelin maturation (5,13) suggests that specific molecular species are required to support the characteristic functions of this membrane. However, the analysis of total fatty acids alone cannot describe the changes in the distribution of myelin phospholipid molecular species during development. Therefore, a study was undertaken to determine the PtdCho, phosphatidylethanolamine (PtdEtn) and PlsEtn molecular species in rat brain myelin in the course of maturation.

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Abbreviations: HPLC, high-performance liquid chromatography; PlsEtn, plasmenylethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Isolation of rat brain myelin. Rats were bred in our own animal facility. Before being used in this study, rats had been fed for several generations on a pelleted diet containing linoleic and linolenic acids (54.5 and 4.2% by weight of the lipid fraction, respectively) as the only polyunsaturated fatty acids. Whole brains of 15- or 21-day-old or adult rats were excised and homogenized in 0.32 M sucrose, and the myelin was isolated according to the method by Norton and Poduslo (8) adapted for different ages. To isolate myelin from 15- and 20-day-old rats involved keeping the amount of brain per tube constant (approximately 5%, wt/vol) in the first step to minimize contamination. With this modification, preparations of similar purity (at least 95% pure) were obtained at all ages examined (8). The myelin fractions were stored after lyophilization at −80°C until use.

Extraction and characterization of phospholipid molecular species. Lipids from lyophilized myelin were extracted and partitioned according to the procedure of Folch *et al.* (14). Choline and ethanolamine phospholipids were separated by one-dimensional thin-layer chromatography (TLC) after purification by silicic acid column chromatography (15).

The molecular species composition of each phospholipid subclass was determined by high-performance liquid chromatography (HPLC) of the dinitrobenzoyl derivatives. Briefly, diradylglycerols were prepared by hydrolysis of each phospholipid class with phospholipase C from *Bacillus cereus* (Sigma Chemical Co., St. Louis, MO) and then converted to their dinitrobenzoyl derivatives as reported by Kito *et al.* (16) and Francescangeli *et al.* (17). The diacyl-, alkylacyl- and alkenylacylglycerol derivatives were separated by TLC (Silica Gel G60 plates; Merck, Darmstadt, Germany) using benzene as solvent (R_f values of 0.23, 0.39 and 0.56 respectively; Ref. 18). The molecular species of each subclass were eluted with

hexane/diethyl ether (1:1, vol/vol) from the silica gel and characterized and quantified by HPLC (column 4.6 mm × 15 cm Resolve C_{18}, 5 μm; Waters Cidra, Puerto Rico), using acetonitrile as solvent at a flow rate of 2 mL/min at 13°C and ultraviolet detection at 230 nm. Data were processed with a Spectra physics SP 4290 integrator (San Jose, CA). Individual molecular species were identified as reported by Leray *et al.* (18) by gas–liquid chromatography (15), using various diacylglycerol species from commercial sources (Larodan Fine Chemicals, Malmö, Sweden) as standards and by developing a RRT/carbon number plot as previously described (19,20).

The phospholipid composition of myelin was assessed by phosphorus analysis (21) after separating phospholipids by two-dimensional TLC (22).

RESULTS

Phospholipid composition of rat myelin during development. The phospholipid composition of the myelin isolated from rat brain at different ages is presented in Table 1. The data show that between the 15th and 90th day the percentages of PtdCho and PtdEtn decreased by about 18 and 62%, respectively. This decrease was compensated for by a large increase (about 90%) in PlsEtn levels whereas the percentages of the other phospholipid did not change significantly.

Molecular species of choline and ethanolamine phospholipids. Molecular species analysis of PtdCho, PtdEtn and PlsEtn revealed that each of these classes contained about 17 well-defined molecular species (Fig. 1). In myelin of 15-day-old rats, each class had a specific pattern of molecular species that changed in the course of development.

PtdCho. In myelin of young rats, the saturated/monounsaturated species and specifically 16:0/18:1n-9 and 18:0/18:1n-9 (Table 2) amounted to about 55%. The content of saturated/monounsaturated species increased in adult myelin to about 70% resulting from the increase in 18:0/18:1n-9 (from 18 to 33%) while the 16:0/18:1n-9 levels remained unchanged. PtdCho also contained a large amount (about 24%) of the disaturated species 14:0/16:0, 16:0/16:0 and 16:0/18:0, which decreased between 2- and

TABLE 1

Phospholipid Composition of Brain Myelin During Development[a]

	Age		
	15 days	20 days	Adult
PtdCho	35.15 ± 1.16	35.89 ± 1.51	28.81 ± 1.05
PtdEtn	27.11 ± 1.79	15.25 ± 2.18	10.24 ± 2.02
PlsEtn	17.06 ± 1.24	19.21 ± 1.61	32.41 ± 2.61
PtdSer	11.25 ± 0.37	10.62 ± 0.59	11.77 ± 0.41
PtdIns	1.33 ± 0.17	3.85 ± 0.77	2.50 ± 0.62
PtdOH	1.93 ± 0.07	2.79 ± 0.14	2.39 ± 0.12
Cer*P*Cho	5.56 ± 0.13	6.89 ± 0.68	6.03 ± 0.77

[a]The phospholipid composition is expressed in mol%. The values are the means ± SD of duplicate determinations from three different experiments. Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PlsEtn, plasmenylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; Cer*P*Cho, sphingomyelin.

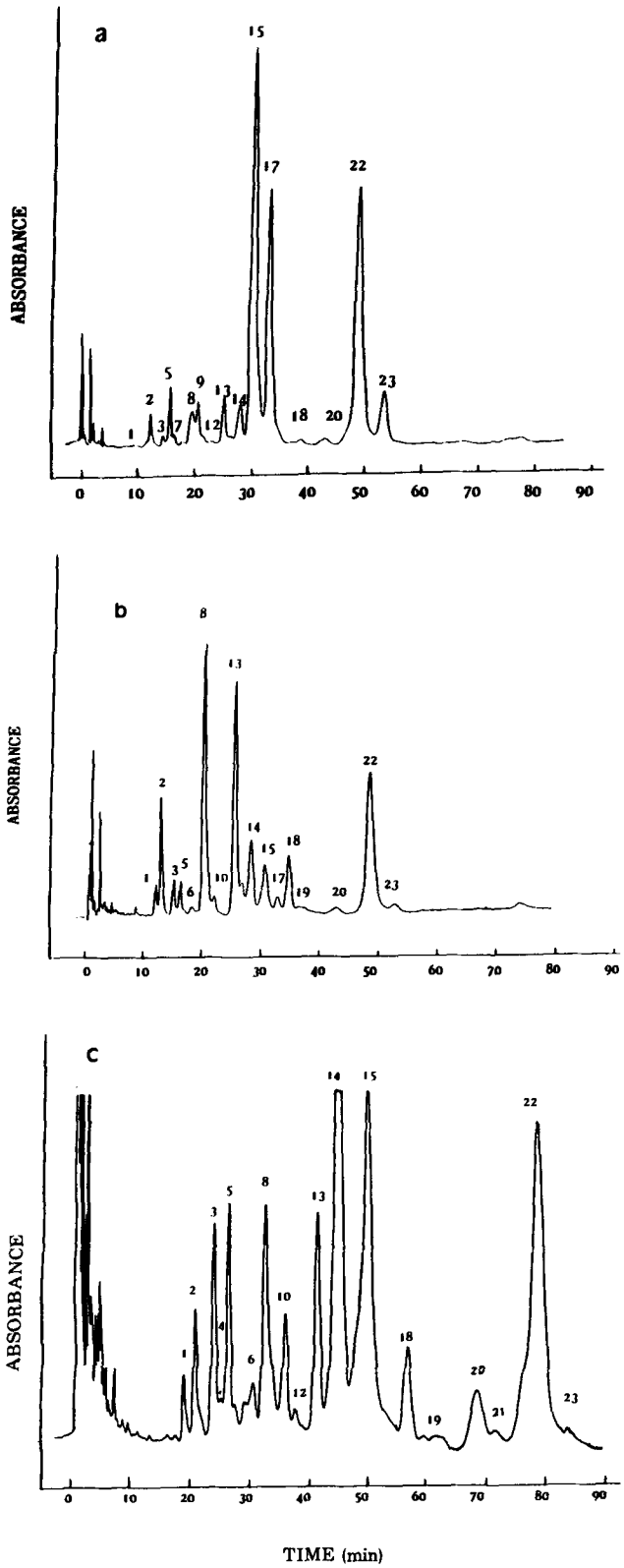

FIG. 1. High-performance liquid chromatography (HPLC) separation of molecular species of dinitrobenzoylated diradylglycerols obtained from a) phosphatidylcholine, b) phosphatidylethanolamine and c) plasmenylethanolamine of 1-month-old rat brain myelin. Phospholipids were purified, derivatized and subjected to reverse-phase HPLC as described under Material and Methods. Detection was by absorption at 230 nm. The peak numbers corresponds to those in Tables 2 to 4.

TABLE 2

Phosphatidylcholine Molecular Species of Rat Brain Myelin During Development[a]

Peak number	Molecular species	Age		
		15 days	20 days	Adult
1	18:1/22:6n-3	0.60 ± 0.01	0.63 ± 0.03	0.10 ± 0.01
2	16:0/22:6n-3	1.57 ± 0.37	1.90 ± 0.09	1.10 ± 0.22
3	18:1/20:4n-6	0.73 ± 0.10	0.67 ± 0.10	0.60 ± 0.01
4	16:0/22:5n-3	—[b]	—	—
5	16:0/20:4n-6	3.33 ± 0.07	2.33 ± 0.05	1.67 ± 0.05
6	18:1/18:2n-6	0.71 ± 0.01	0.80 ± 0.05	0.50 ± 0.01
7	16:0/18:2n-6	2.57 ± 0.03	1.03 ± 0.07	2.23 ± 0.61
8	18:0/22:6n-3	2.03 ± 0.03	3.03 ± 0.47	0.77 ± 0.35
9	14:0/16:0	3.60 ± 0.09	1.37 ± 0.27	0.47 ± 0.20
10	16:0/22:4n-6	—	—	—
11	16:0/20:3n-6	—	—	—
12	18:0/22:5n-3	—	—	—
13	18:0/20:4n-6	5.40 ± 0.34	4.50 ± 0.21	3.57 ± 0.17
14	18:1/18:1n-9	4.43 ± 0.12	5.03 ± 0.07	4.63 ± 0.31
15	16:0/18:1n-9	34.63 ± 0.67	34.13 ± 0.22	36.67 ± 0.41
16	18:0/18:2n-6	—	—	—
17	16:0/16:0	16.17 ± 0.40	12.53 ± 0.03	8.30 ± 0.79
18	18:0/22:4n-6	0.57 ± 0.05	0.90 ± 0.24	0.83 ± 0.09
19	18:0/20:3n-6	—	—	—
20	18:1/20:1n-9	0.77 ± 0.07	1.20 ± 0.03	1.10 ± 0.09
21	16:0/20:1n-9	0.19 ± 0.01	0.23 ± 0.03	1.83 ± 0.39
22	18:0/18:1n-9	18.27 ± 0.30	24.33 ± 0.35	33.00 ± 0.75
23	16:0/18:0	4.10 ± 0.17	4.57 ± 0.03	1.97 ± 0.23

[a]Results are expressed in mol% and represent the mean values ± SD of duplicate determinations from three different experiments.
[b]—, Not detected.

7-fold in adult myelin. The polyunsaturated molecular species amounted to only about 17% in myelin of 15-day-old rats, the major molecular species being 16:0/22:6n-3, 18:0/22:6n-3, 16:0/20:4n-6 and 18:0/20:4n-6. The percentage of the saturated/polyunsaturated species decreased about 1.5- to 2.0-fold in adult myelin.

PtdEtn. In contrast to PtdCho, the PtdEtn of 15-day-old rat brain myelin contained much less saturated/monounsaturated molecular species (about 27%) and only very low amounts of saturated/saturated species (about 5%) (Table 3). About 58% of the molecular species were saturated/polyunsaturated, including 18:0/22:6n-3 and 18:0/20:4n-6. This subclass also contained noticeable amounts of 18:0/22:4n-6, which was essentially absent in PtdCho. During development, a 2- and 5-fold decrease, respectively, in the percentages of 22:6n-3 containing molecular species and in disaturated species was observed whereas species containing 20:4n-6 remained unchanged while the percentage of 18:0/22:4n-6 nearly doubled. Among the saturated/monounsaturated species, only 18:0/18:1n-9 increased (about 1.8-fold) whereas 16:0/18:1n-9 decreased by approximately 30%. No significant changes were observed in dimonounsaturated molecular species.

PlsEtn. This subclass was characterized by the absence of saturated/saturated species (Table 4). About 60% of the molecular species contained polyunsaturated fatty acids in position-2 of the glycerol, mainly 22:6n-3 and 20:4n-6, but also noticeable amounts of 22:5n-3 and 22:4n-6. The saturated/monounsaturated species accounted for about 25% (mainly 16:0/18:1n-9, 18:0/18:1n-9) with appreciable amounts of dimonounsaturated species (about 15%), mainly 18:1/18:1n-9 and 18:1/20:1n-9. The maturation of myelin was associated with a 2- to 4-fold decrease in molecular species containing 22:6n-3 and 20:4n-6 except for 18:1/20:4n-6, which remained constant. This decrease was compensated for by a 2- to 4-fold increase in saturated/monounsaturated molecular species except for 16:0/18:1n-9 which did not change significantly. In contrast to PtdCho and PtdEtn, the dimonounsaturated species (18:1/18:1n-9 and 18:1/20:1n-9) in PlsEtn increased significantly (about 2- to 3-fold).

DISCUSSION

Myelinogenesis begins in rat brain at about the 10th day after birth. The maximum rate of myelination occurs between the 15th and 30th day after birth, but myelination continues at a lower rate throughout life. Earlier studies showed that although the proportion of total protein to total lipids appears to be relatively constant at all ages, the myelin in brains of young animals has a different composition than that of adults. Major changes are the increase in total galactolipids and the decrease in total phospholipids (5). Our data show a decrease in PtdCho and PtdEtn with age, which is compensated for by an increase in PlsEtn. The percentage of the other phospholipids does not change significantly. These observations are in agreement with those reported by Horrocks (7) and Eng and Noble (9), and it was suggested that the molecular species of the various phospholipids may also undergo developmental changes. The findings of a decrease in polyunsaturated fatty acids and an increase in monoenoic acids in the various myelin phospholipid subclasses are consistent with this hypothesis. However, until now no conclusions could be drawn as to the type of molecular species

TABLE 3

Phosphatidylethanolamine Molecular Species of Rat Brain Myelin During Development[a]

Peak number	Molecular species	Age		
		15 days	20 days	Adult
1	18:1/22:6n-3	2.40 ± 0.08	2.03 ± 0.14	1.07 ± 0.10
2	16:0/22:6n-3	7.07 ± 0.24	5.53 ± 0.34	3.20 ± 0.05
3	18:1/20:4n-6	3.00 ± 0.08	3.37 ± 0.33	3.97 ± 0.15
4	16:0/22:5n-3	—[b]	—	—
5	16:0/20:4n-6	2.83 ± 0.03	2.23 ± 0.19	2.87 ± 0.03
6	18:1/18:2n-6	0.47 ± 0.03	1.37 ± 0.15	1.40 ± 0.34
7	16:0/18:2n-6	—	—	—
8	18:0/22:6n-3	17.30 ± 0.50	18.13 ± 0.54	11.47 ± 0.63
9	14:0/16:0	—	—	—
10	16:0/22:4n-6	1.73 ± 0.12	1.47 ± 0.22	1.33 ± 0.10
11	16:0/20:3n-6	—	—	—
12	18:0/22:5n-3	—	—	—
13	18:0/20:4n-6	18.47 ± 0.51	16.00 ± 0.69	18.00 ± 0.54
14	18:1/18:1n-9	8.63 ± 0.23	10.03 ± 0.64	9.77 ± 0.30
15	16:0/18:1n-9	11.43 ± 0.75	9.93 ± 1.71	8.00 ± 0.26
16	18:0/18:2n-6	—	1.27 ± 0.14	1.03 ± 0.15
17	16:0/16:0	3.97 ± 0.60	3.43 ± 0.60	0.73 ± 0.17
18	18:0/22:4n-6	4.63 ± 0.12	4.77 ± 0.33	8.53 ± 2.03
19	18:0/20:3n-6	—	—	—
20	18:1/20:1n-9	1.17 ± 0.03	1.90 ± 0.05	2.57 ± 0.10
21	16:0/20:1n-9	—	—	0.97 ± 0.19
22	18:0/18:1n-9	14.80 ± 0.05	17.07 ± 0.52	26.23 ± 1.99
23	16:0/18:0	1.37 ± 0.17	1.17 ± 0.07	—

[a]Results are expressed in mol% and represent the means values ± SD of duplicate determinations from three different experiments.
[b]—, Not detected.

TABLE 4

Plasmenylethanolamine Molecular Species of Rat Brain Myelin During Development[a]

Peak number	Molecular species[b]	Age		
		15 days	20 days	Adult
1	18:1/22:6n-3	4.10 ± 0.08	4.03 ± 0.07	1.10 ± 0.05
2	16:0/22:6n-3	7.40 ± 0.54	5.23 ± 0.07	1.60 ± 0.08
3	18:1/20:4n-6	4.70 ± 0.33	5.13 ± 0.14	5.37 ± 0.33
4	16:0/22:5n-3	1.93 ± 0.29	0.90 ± 0.33	1.87 ± 0.33
5	16:0/20:4n-6	6.13 ± 0.40	4.00 ± 0.05	1.87 ± 0.47
6	18:1/18:2n-6	—[c]	—	—
7	16:0/18:2n-6	—	—	—
8	18:0/22:6n-3	14.23 ± 1.35	13.00 ± 0.53	8.00 ± 0.21
9	14:0/16:0	—	—	—
10	16:0/22:4n-6	5.47 ± 0.12	4.67 ± 0.26	2.47 ± 0.17
11	16:0/20:3n-6	1.25 ± 0.20	2.40 ± 0.57	—
12	18:0/22:5n-3	2.50 ± 0.80	1.87 ± 0.83	1.30 ± 0.35
13	18:0/20:4n-6	7.87 ± 0.31	6.97 ± 0.03	4.77 ± 0.11
14	18:1/18:1n-9	11.33 ± 0.44	15.00 ± 0.42	30.43 ± 1.03
15	16:0/18:1n-9	11.77 ± 0.80	13.63 ± 0.44	14.07 ± 0.29
16	18:0/18:2n-6	—	—	—
17	16:0/16:0	—	—	—
18	18:0/22:4n-6	4.03 ± 0.21	4.23 ± 0.11	2.67 ± 0.03
19	18:0/20:3n-6	1.50 ± 0.25	1.70 ± 0.05	0.50 ± 0.11
20	18:1/20:1n-9	3.53 ± 0.21	4.17 ± 0.15	7.30 ± 0.29
21	16:0/20:1n-9	0.73 ± 0.19	0.63 ± 0.23	3.17 ± 0.15
22	18:0/18:1n-9	9.63 ± 0.77	13.47 ± 0.48	14.27 ± 1.18
23	16:0/18:0	—	—	—

[a]Results are expressed in mol% and represent the mean values ± SD of duplicate determinations from three different experiments.
[b]The vinyl bond is not counted when listing double bonds of the alk-1-enyl chain located in position 1.
[c]—, Not detected.

involved in these developmental changes, since phospholipid fatty acid composition alone does not indicated the combinations of fatty acids that are present in a particular phospholipid.

In rat brain myelin, each phospholipid subclass showed a specific molecular species composition (Tables 2–4) as had been shown for whole brain (23) and cerebellum (18).

Although specific changes were observed in each phospholipid subclass, the major changes were the marked increase in only the 18:0/18:1n-9 species in PtdCho and PtdEtn and in the 18:0/18:1n-9 and 18:1/18:1n-9 species in PlsEtn, confirming our hypothesis that these two molecular species are preferentially located in the myelin sheaths (18). The increases were mainly compensated for by a decrease in disaturated and/or polyunsaturated fatty acid containing species. Ousley and Morell (24) reported that in rat myelin the different molecular species of choline and ethanolamine phospholipids turned over at different rates, and that for both phospholipids the 18:0/18:1 species had the slowest turnover. It thus becomes clear that, during maturation, myelin becomes enriched in phospholipid molecular species with slow turnovers, indicating that myelin may require specific phospholipids to maintain function.

One of the major issues raised by the present observations concerns the mechanism by which the specific phospholipid composition of the myelin and changes during maturation may be controlled. The assembly of the phospholipids in myelin involves the synthesis of the various molecular species, their transport to the membrane, their sorting and insertion into the membrane, as well as subsequent fatty acid remodelling. The addition of newly synthesized lipids to myelin does not depend on the addition of newly synthesized protein (25). Thus, the assembly of specific phospholipids in myelin is not only controlled by the mechanisms that regulate their insertion into this membrane, but also by the mechanisms that regulate their synthesis, their transport and the remodelling of their fatty acid composition.

The *de novo* synthesis of phospholipids occurs in the endoplasmic reticulum of the oligodendroglial cells which is followed by transport to the plasma membrane. In this respect, the observations that the synthesis of polyunsaturated fatty acids decreases greatly, whereas that of saturated and monounsaturated fatty acids is maximal during intense myelination (26), suggest that the increase in 18:0/18:1n-9 phospholipids in myelin may be controlled at the site of their synthesis and not by lipid transfer proteins involved in their transport. However, this would not explain the different phospholipid molecular species composition in the various intracellular membranes. Therefore it seems likely that the specific assembly of phospholipid molecules in myelin may also be controlled by mechanisms involved in the sorting and integration of the various molecular species. It appears likely that various enzymes of phospholipid metabolism participate in this control. The presence of such enzymes in myelin sheaths, including phospholipases (27,28), acyl-CoA:lysophospholipid acyltransferases (29), acyl-CoA:synthetase (30) and choline and ethanolamine phosphotransferases (31), is consistent with this hypothesis. These enzymes would be expected to participate in the remodelling of certain phospholipid molecules in the myelin in the course of maturation.

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ARTICLE

Synthesis of a Phosphatidyl Derivative of Vitamin E and Its Antioxidant Activity in Phospholipid Bilayers

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A novel phospholipid containing a chromanol structure at its polar head group was synthesized from egg yolk phosphatidylcholine and 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman by transphosphatidylation catalyzed by phospholipase D from *Streptomyces lydicus*. The structure of the product synthesized was shown by spectral analysis to be 1,2-diacyl-*sn*-glycero-3-phospho-2'-hydroxyethyl-2',5',7',8'-tetramethyl-6'-hydroxychroman. The phosphatidylchromanol (PCh) showed antioxidant activity against radical chain oxidation of methyl linoleate in solution in a manner similar to that of *d*-α-tocopherol (α-Toc) and 2,2,5,7,8-pentamethyl-6-chromanol. However, PCh was less effective as a chain-breaking antioxidant than was α-Toc when unilamellar egg yolk phosphatidylcholine liposomes were exposed to either a water-soluble or a lipid-soluble radical initiator. It is likely that the phospholipid nature of PCh affects the location and the mobility of the chromanol moiety in the membrane bilayer resulting in a decrease in antioxidant activity. On the other hand, the antioxidant activity of PCh was little different from that of α-Toc in unilamellar liposomes when exposed to a lipid-soluble radical initiator in the presence of ascorbic acid. It appears that PCh in phospholipid bilayers can be regenerated by ascorbic acid in aqueous phase as can be α-Toc. The new phospholipid, phosphatidylchromanol, should prove useful as a chain-breaking antioxidant in phospholipid membranes. *Lipids* 29, 83–89 (1994).

Lipid peroxidation in biomembranes contributes significantly to oxidative damage in biological systems (1). It is also known that biomembranes possess an antioxidant defense against lipid peroxidation (2), in which *d*-α-tocopherol (α-Toc) is thought to act as a potent chain-breaking reagent (3). The mechanism of radical scavenging by α-Toc in homogenous solution is well understood (4,5). It is also known that α-Toc prevents phospholipid peroxidation in biomembranes (6–8). Although a number of studies have been undertaken using liposomes as a membrane model (6–10), the mechanism of antioxidant action of α-Toc in biomembranes is still not entirely clear. In particular, the location in the phospholipid bilayer of the chromanol ring, which is the active moiety of α-Toc for radical-scavenging that interferes with the reaction with chain-propagating lipid peroxyl radicals, is still a subject of discussion (10–13).

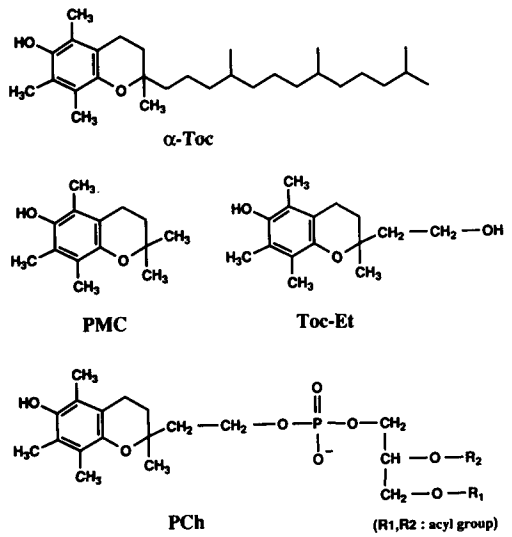

FIG. 1. Molecular structures of *d*-α-tocopherol, (α-Toc), 2,2,5,7,8-pentamethyl-6-chromanol (PMC), 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman (Toc-Et) and phosphatidylchromanol (PCh).

The transphosphatidylation activity of phospholipase D (PLD) is commonly used to modify the polar head group of phospholipids (14,15). We previously described the synthesis of 6-phosphatidyl-L-ascorbic acid catalyzed by PLD (16). This phosphatidyl derivative of ascorbic acid was shown to prevent liposomal lipid oxidation more effectively than ascorbic acid (17). This, in turn, prompted us to synthesize a phospholipid containing a chromanol ring, as such an α-Toc analogue would seem helpful in investigating the antioxidant action of α-Toc in membranes.

In the present work, we report on the synthesis of a new phospholipid containing a chromanol ring (Fig. 1), as well as on the antioxidant activity of the phosphatidylchromanol (PCh) synthesized in unilamellar liposomes and in hexane/isopropanol solution.

MATERIALS AND METHODS

Chemicals. Phosphatidylcholine (PC) from egg yolk and dimyristoyl PC were purchased from Sigma Chemical Co. (St. Louis, MO). *d*-α-Toc and 2,2,5,7,8-pentamethyl-

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Abbreviations: AAPH, 2,2'-azo*bis*(2-amidinopropane) hydrochloride; AMVN, 2,2'-azo*bis*(2,4-dimethylvaleronitrile); AsA, ascorbic acid; DTPA, *diethylenetriaminepentaacetic acid*; HPLC, high-performance liquid chromatography; LUV, large unilamellar vesicles; MeL-OOH, methyl linoleate hydroperoxides; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PCh, phosphatidylchromanol; PC-OOH, phosphatidylcholine hydroperoxides; PLD, phospholipase D; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; SIMS, sputtered ion mass spectrometry; TLC, thin-layer chromatography; α-Toc, *d*-α-tocopherol; Toc-Et, 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman.

6-chromanol (PMC) (Fig. 1) were obtained from Eisai Co. (Tokyo, Japan). 2,5,7,8-Tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman (Toc-Et) was kindly supplied by Kuraray Co. Ltd. (Kurashiki, Japan) (Fig. 1). PLD from *Streptomyces lydicus* was kindly supplied by Honen Co. (Yokohama, Japan). 2,2'-Azo*bis*(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azo*bis*(2,4-dimethylvaleronitrile) (AMVN) were obtained from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals used were of reagent grade.

Preparation of PCh. PCh was synthesized by reacting 2.5 mL of 25 mM egg yolk PC and 25 mM Toc-Et in diethyl ether and 25 mL of 10 U of PLD and 10 mM $CaCl_2$ in 10 mM acetate buffer (pH 5.1) at 37°C with continuous shaking. After 2 h of incubation, 3 mL of 6 N HCl was added, and the product was extracted three times with 30 mL of chloroform/methanol (2:1, vol/vol). The combined chloroform layers were washed with 20 mL of water, and evaporated in a rotary evaporator. The residue was dissolved in chloroform/methanol (95:5, vol/vol) at 10 mg/mL and placed onto a silica gel column (Lichroprep Si 60 40–63 μm; Merck, Darmstadt, Germany) that had previously been equilibrated with the same solvent. The product was eluted at a flow rate of 1.8 mL/min, 1.0-mL fractions were collected, and fractions were monitored by thin-layer chromatography (TLC) (Silica gel 60; Merck) using chloroform/methanol (8:2, vol/vol) as developing solvent. Phospholipids were visualized by spraying with Dittmer's reagent (18). Reducing activity in TLC fractions was detected by exposure to ferric ion reagent (19). Fractions containing PCh were redissolved in methanol, and then stored at –20°C until use.

Assay of PCh and phosphatidic acid (PA) in the reaction mixture. PCh and PA were assayed by high-performance liquid chromatography (HPLC) on an aminopropane-bonded silica column (Shim-Pack FLC-NH_2, 4.6 × 50 mm, 3 μm; Shimadzu Co., Kyoto, Japan) using *n*-hexane/isopropanol/10% phosphoric acid (70:30:0.5, by vol) at a flow rate of 2.0 mL/min. PCh and PA eluted at 1.5 and 2.2 min, and were monitored at 295 and 206 nm, respectively.

Spectral analyses. Infrared spectra were measured on KBr pellets using a JASCO FT/IR-7300 infrared spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Mass spectra were measured in the sputtered ion mass spectrometry (SIMS) mode using a Hitachi M-80B instrument (Hitachi Co., Tokyo, Japan). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured with a JEOL GSX-270 FT NMR spectrometer (Japan Electronic Optics Laboratory Co., Tokyo, Japan) at 270 and 67.9 MHz, respectively. Samples were dissolved in $CDCl_3/CD_3OD$ (2:1, vol/vol), and tetramethylsilane was used as internal standard.

Peroxyl radical-scavenging activity in solution. Peroxyl radical-scavenging activities of α-Toc, PMC and PCh in homogeneous solution were determined by measuring the inhibition of free radical oxidation of methyl linoleate that was initiated by a lipid-soluble azo compound (17,20). The reaction mixture contained 110 μmol of methyl linoleate and 0.1 μmol of antioxidant in 1.0 mL of *n*-hexane/isopropanol (7:3, vol/vol). Oxidation was initiated by adding 110 mM AMVN (100 μL) in the same solvent. The mixture was incubated at 37°C with continuous shaking in the dark and aliquots were withdrawn at regular intervals to measure methyl linoleate hydroperoxides (MeL-OOH) (21).

Preparation of unilamellar liposomes. Liposomes [large unilamellar vesicles (LUV)] were prepared by an extrusion method (22). First, PC was purified to remove contaminant peroxides by reverse-phase column chromatography as described previously (23). A stock solution of purified PC in chloroform, with or without antioxidants, was placed into a test tube and was evaporated under a stream of nitrogen, then *in vacuo.* The thin lipid film on the glass wall was dispersed with a vortex mixer for 1 min in 0.7 mL Tris-HCl buffer (0.01 M, pH 7.4) containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) followed by ultrasonic irradiation with a Heat Systems Sonifier (Model W-380; Farmingdale, NY) for 30 s. LUV were obtained by extruding the suspension through polycarbonate filters mounted in an extrusion apparatus (LiposoFast, Avestin, Inc., Ottawa, Canada), and the LUV were diluted with the same volume of Tris-buffer.

Lipid peroxidation of LUV initiated by radical initiators. In the experiments with a water-soluble radical initiator, oxidation was started by the addition of AAPH to the LUV suspension. Oxidation was carried out in the dark at 37°C under air with continuous shaking. In the experiments with lipid-soluble radical initiator, AMVN was mixed with PC before the preparation of the liposomes, and the reaction mixture was kept at 50°C during the oxidation. Aliquots (10 μL) were withdrawn at specific time intervals and phosphatidylcholine hydroperoxides (PC-OOH) were quantified by HPLC as described previously (24). The octane-bonded silica column (TSK-gel OCTYL-80TS column, 6 × 150 mm, 5 μm; TOSOH, Tokyo, Japan) was eluted with methanol/water (93.5:6.5, vol/vol). Standard PC-OOH was prepared as described (24).

Determination of antioxidants during liposomal peroxidation. α-Toc, PMC and PCh were measured by HPLC on an octodecane-bonded silica column (YMC-pack ODS, 6 × 150 mm, 5 μm; Yamamura Chemical Laboratories, Kyoto, Japan) that was eluted with acetonitrile/ethanol (60:40, vol/vol) at a flow rate of 2.5 mL/min. The eluent was monitored fluorometrically at an excitation wavelength of 298 nm and an emission wavelength of 325 nm using a JASCO Spectrofluorometer 821-FP (Tokyo, Japan). α-Toc, PMC and PCh were eluted at 4.5, 1.5 and 1.7 min, respectively. Ascorbic acid (AsA) was assayed by HPLC using a cation exchange column (TSK gel SCX, 7.8 × 300 mm, 5 μm, TOSOH) with 2 mM phosphoric acid as eluent at a flow rate of 1.2 mL/min. The eluent was monitored at 254 nm; AsA was eluted at 6.6 min.

RESULTS

Synthesis of PCh. The time course of the enzymatic reaction for the synthesis of PCh catalyzed by PLD is

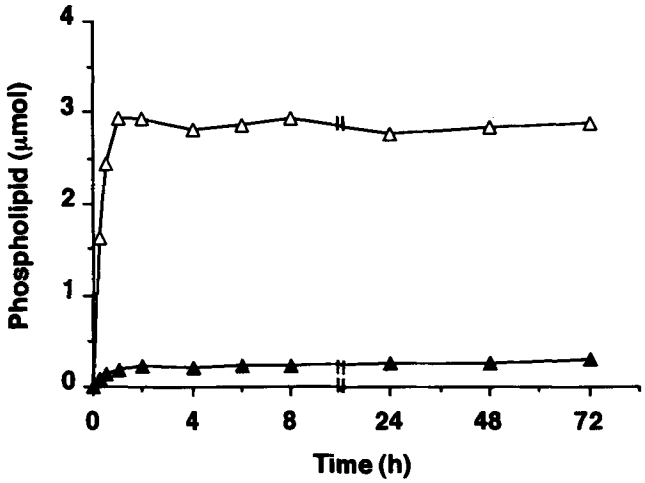

FIG. 2. Time course for the synthesis of phosphatidylchromanol catalyzed by phospholipase D. The amounts of PCh and phosphatidic acid (PA) were calculated from high-performance liquid chromatography peak areas using calibration curves; (△), PCh; (▲), PA.

shown in Figure 2. The reaction reached a plateau after 1.5 h of incubation, and PC was converted to the new product in high yield (approximately 94%). Little PA accumulated throughout the reaction period, indicating that little hydrolysis occurred. The new product gave a blue spot on TLC with Dittmer reagent and a red spot with ferric ion reagent, indicating that the new compound was a phospholipid with reducing activity.

Spectral analyses were carried out to identify the structure of the phospholipid synthesized from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine. The SIMS spectrum showed an [M + H^+] ion at *m/z* 825. The infrared spectrum indicated the presence of a phosphate group and of hydroxyl at the chromanol ring (KBr, cm^{-1} : 1745, C=O; 1457,–OH; 1246, P=O; 1027, P-O-C). To elucidate the structure in detail, ^{13}C NMR chemical shifts were assigned to the carbons of the dimyristoylglycerophosphate and the chroman ring moieties (Table 1). The ^{13}C chemical shifts of the dimyristoylglycerol moiety of the product were essentially the same as those of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (16). The ^{13}C chemical shifts of the chroman ring moiety were essentially the same as those of Toc-Et except for the C-1' and C-2' shifts. The C-2' signal was shifted downfield (3.24 ppm) relative to that of Toc-Et, while the C-1' signal was shifted slightly upfield (1.43 ppm). Such ^{13}C chemical shifts for carbons proximal to phosphate were also observed in the spectra of 6-phosphatidyl-L-ascorbic acid (16) and other phosphoesters (14). Based on these data and on ^1H NMR spectra (data not shown), the compound was identified as 1,2-dimyristoyl-*sn*-glycero-3-phospho-2'-hydroxyethyl-2',5',7',8'-tetramethyl-6'-hydroxy-chroman.

Peroxyl radical-scavenging activity of PCh, α-Toc and PMC in solution. Peroxyl radical-scavenging activities of PCh and of related compounds were evaluated by measuring the inhibition of methyl linoleate peroxidation initiated by AMVN in hexane/isopropanol solution. A

TABLE 1

^{13}C NMR Spectra of PCh, Dimyristoyl PC and Toc-Et[a]

	Chemical shifts (δ)		
Carbons	PCh	Dimyristoyl PC[b]	Toc-Et
Dimyristoyl glycerol moiety			
CH_2O (*sn*-1)	62.85	63.01	
CHO (*sn*-2)	70.78	70.74	
CH_2OP (*sn*-3)	63.86	63.93	
CO ester	174.27	174.30	
	173.90	173.93	
$(CH_2)_n$	34.48–22.99	34.55–22.96	
CH_3 terminal	14.22	14.21	
Choline moiety			
CH_2OP		59.34	
CH_2N		66.79	
$(CH_3)_3N$		54.39	
Toc-Et moiety			
C-2	73.63		75.49
C-3	32.40		31.85
C-4	20.92		20.51
C-5	117.25		118.72
C-6	145.40		144.72
C-7	120.93		121.20
C-8	122.51		122.35
C-2a	23.92		23.04
C-4a	123.45		117.15
C-5a	11.60		11.27
C-7a	11.91		12.05
C-8a	12.56		12.05
C-8b	145.40		145.09
C-1'	40.53		41.96
C-2'	62.49		59.25

[a]Chemical shifts are given downfield from tetramethylsilane.
[b]Data for dimyristoyl PC are those reported by Nagao *et al.* (16).
Abbreviations: NMR, nuclear magnetic resonance; PCh, phosphatidylchromanol; Dimyristoyl PC, 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine; Toc-Et, 2, 5, 7, 8-tetramethyl-6-hydroxy-2-(hydroxyethyl) chroman.

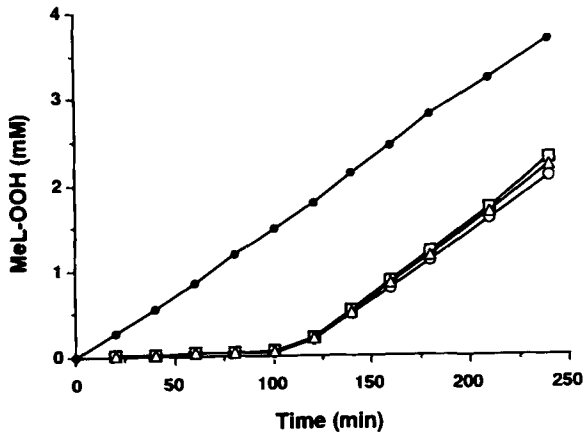

FIG. 3. Inhibition of the 2,2'-azo*bis*(2,4-dimethylvaleronitrile) (AMVN)-initiated oxidation of methyl linoleate in solution by α-Toc, PMC and PCh. The reaction system consisted of methyl linoleate (100 mM), antioxidant (90.9 μM) and AMVN (10 mM) in *n*-hexane/isopropanol (7:3, vol/vol); (●), no addition; (○), α-Toc; (□), PMC; (△), PCh. See Figure 1 for other abbreviations; MeL-OOH, methyl linoleate hydroperoxides.

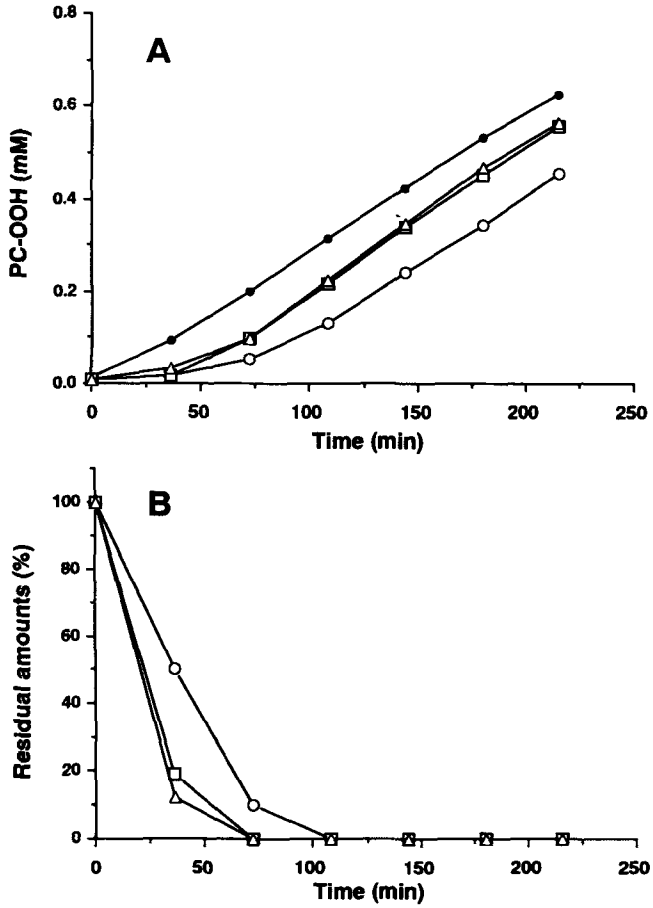

FIG. 4. Accumulation of phosphatidylcholine hydroperoxides (PC-OOH) (A) and loss of α-Toc, PMC and PCh (B) during phospholipid peroxidation in large unilamellar vesicles exposed to 2,2'-azo*bis*(2-amidinopropane)hydrochloride (AAPH). The reaction system consisted of PC (5 mM), α-Toc, PMC or PCh (10 μM), diethylenetriaminepentaacetic acid (0.5 mM) and AAPH (10 mM) in 1.1 mL of Tris-HCl buffer; (●), no addition; (○), α-Toc; (□), PMC; (△), PCh. See Figure 1 for other abbreviations.

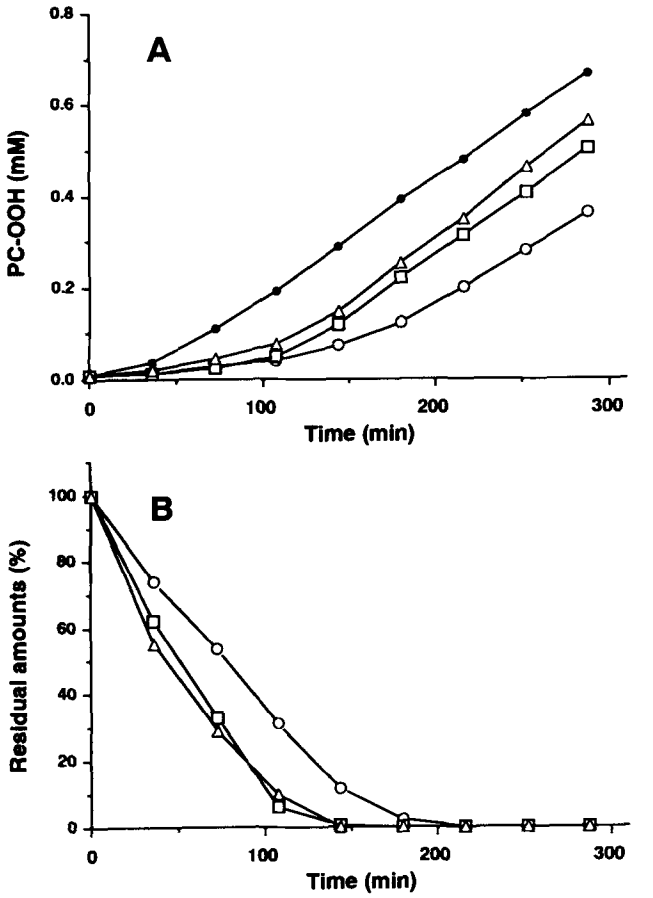

FIG. 5. Accumulation of PC-OOH (A) and loss of α-Toc, PMC and PCh (B) during phospholipid peroxidation in large unilamellar vesicles exposed to AMVN. The reaction system consisted of PC (5 mM), α-Toc, PMC or PCh (5 μM), DTPA (0.5 mM), and AMVN (1 mM) in 1.1 mL of Tris-HCl buffer; (●), no addition; (○), α-Toc; (□), PMC; (△), PCh. See Figures 1, 3 and 4 for abbreviations.

typical example of the inhibition seen is shown in Figure 3. From the data we obtained from three independent experiments, we calculated kinetic parameters by the method of Ingold and colleagues (4,25). PCh, α-Toc and PMC showed almost the same induction periods (t_{inh}; 7190 ± 610 s, 7090 ± 420 s and 7290 ± 490 s for α-Toc, PMC and PCh, respectively). The rates of oxidation (R_{inh}) during the induction period were (9.97 ± 3.16)× 10^{-9} M/s, (4.79 ± 1.56)× 10^{-9} M/s, (5.69 ± 1.02)× 10^{-9} M/s for PCh, α-Toc and PMC, respectively. This indicates that the number of radicals trapped by PCh (n) was the same as that trapped by α-Toc and PMC as n is a function of t_{inh}. The k_{inh}/k_p values, the ratio of the rate constant for the inhibition reaction *vs.* that of the propagation reaction, were calculated from t_{inh} and R_{inh} as described previously (26). For PCh, k_{inh}/k_p was 1480 ± 400, which was slightly lower than for α-Toc (3040 ± 770) and PMC (2520 ± 280).

Free radical-scavenging activity of PCh, α-Toc and PMC in unilamellar liposomes. We used LUV and estimated the antioxidant activities of α-Toc and its analogues against phospholipid peroxidation initiated by free radical generators. Figure 4 shows a typical example of the inhibition of the peroxidation initiated by AAPH by α-Toc and its analogues. The water-soluble radical initiator produces peroxyl radicals in the aqueous phase and thereby attacks phospholipids from the aqueous phase at the membrane surface (27). PC-OOH, the primary products of peroxidation, accumulated in the initial stage of the reaction without antioxidants. α-Toc suppressed the accumulation of PC-OOH with a pronounced induction period (65 min). Although PCh and PMC also retarded lipid peroxidation, their induction periods were shorter than that of α-Toc (36 min for PCh, and 39 min for PMC). In addition, both PCh and PMC were consumed faster than α-Toc.

When we used a lipid-soluble azo compound to initiate peroxyl radical-driven lipid peroxidation within the liposomal membranes, α-Toc showed a longer induction period (126 min) than PCh (94 min) and PMC (97 min), and α-Toc was consumed less rapidly than were the latter two compounds (Fig. 5). Thus, PCh was less effective

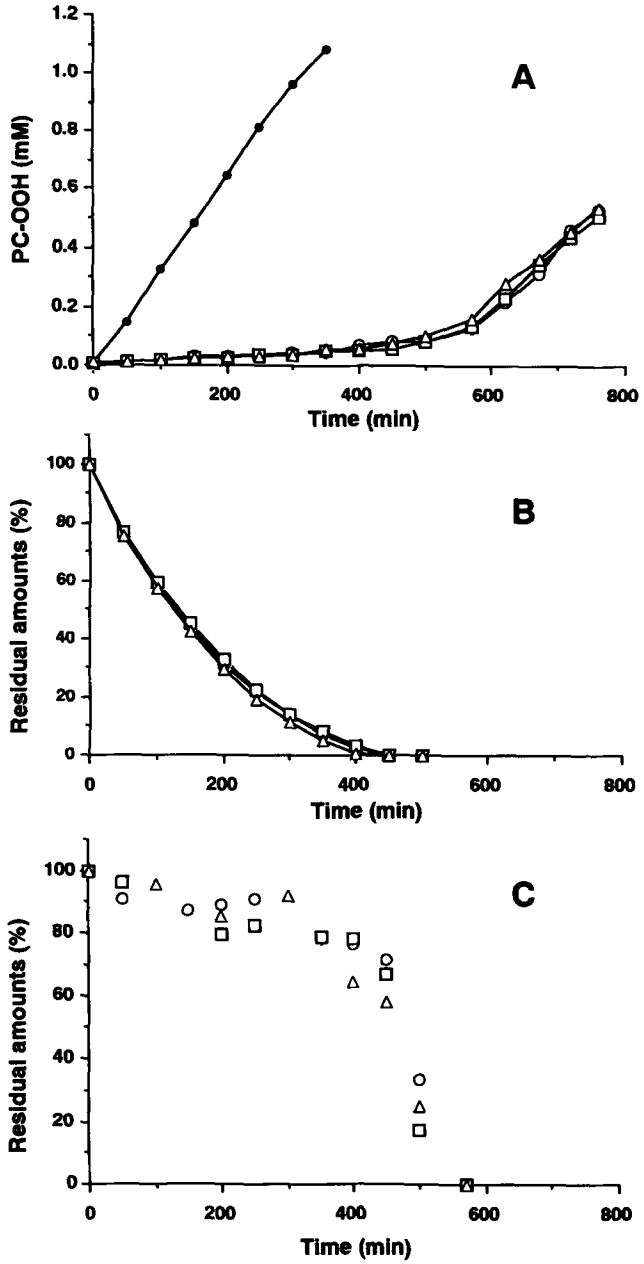

FIG. 6. Accumulation of PC-OOH (A), loss of ascorbic acid (AsA) (B), and loss of α-Toc, PMC or PCh (C) during phospholipid peroxidation in large unilamellar vesicles exposed to AMVN. The reaction system consisted of PC (5 mM), AsA (100 μM), α-Toc, PMC or PCh (5 μM), diethylenetriaminepentaacetic acid (0.5 mM), and AMVN (1 mM) in 1.1 mL of Tris-HCl buffer; (●), no addition; (□), α-Toc; (△), PMC; (○), PCh. See Figures 1, 3 and 4 for abbreviations.

than α-Toc as a chain-breaking antioxidant when either a water-soluble or a lipid-soluble initiator was used to induce the radical chain reaction.

Effect of ascorbic acid on the inhibition by PCh, α-Toc and PMC in liposomal phospholipid peroxidation. Figure 6 shows the inhibition of PC-OOH accumulation by α-Toc or its analogues in the presence of ascorbic acid. It is apparent that the presence of ascorbic acid increased the induction period significantly as indicated by comparison of the data in Figure 6 and Figure 5. Ascorbic acid was consumed in the initial stage, and the levels of α-Toc or its analogues always decreased after ascorbic acid was consumed. Interestingly, there were only small differences in the induction periods and in the consumption of ascorbic acid between the three compounds. Thus, in the presence of AsA PCh exerted the same antioxidant activity as did α-Toc.

DISCUSSION

We previously synthesized phosphatidyl AsA (16) and showed that this phospholipid derivative could be incorporated into phospholipid bilayers, and that it exerted antioxidant activity against oxidative attack from the aqueous phase more efficiently than did ascorbic acid (17). Because vitamin E is a major lipophilic chain-breaking antioxidant in cellular membranes, we now synthesized phosphatidyl vitamin E by the transphosphatidylation catalyzed by PLD. The reaction proceeded with a high yield, and there was little hydrolysis (Fig. 2) as was reported for the hydrolysis of phosphatidylinositol catalyzed by this enzyme (28). Resistance against PLD-catalyzed hydrolysis appears to be due to steric hindrance at the polar head group moiety.

α-Toc consists of two structural domains. One is the chromanol structure responsible for radical scavenging activity and the other is the phytyl side chain which is required for anchoring α-Toc within phospholipid bilayers (29). The product we synthesized by the PLD reaction contains a phospholipid moiety and chromanol moiety, and thus can be considered an α-Toc analogue in which the phytyl side chain is replaced by a phosphatidyl group. The product thus is lipophilic, much like α-Toc. However, introduction of the phosphatidyl group could affect the radical scavenging activity of the chromanol moiety by changing its location in the phospholipid bilayer.

It has already been shown that the phytyl side chain does not affect the free radical scavenging activity of α-Toc in solution (30). Our data show that PMC and α-Toc gave similar kinetic parameters, *i.e.*, k_{inh}/k_p and n values, for free radical oxidation in solution, and this is consistent with the view that the phytyl side chain is not responsible for the inherent antioxidant activity of chromanol. The fact that PCh gave the same n value furthermore suggests that PCh scavenges free radicals by the same mechanism as does α-Toc. The slightly smaller k_{inh}/k_p value for PCh may be due to the lower mobility of PCh in solution or to steric hindrance of scavenging peroxyl radicals as the phospholipids exist in the aggregated form in apolar solvents (31).

Niki and his co-workers (7,10) have shown that the antioxidant effect of α-Toc in phospholipid membranes is lower than in homogeneous solution, and that PMC is more effective than α-Toc in scavenging free radicals in membranes. The higher activity of PMC in membranes could be explained by differences between PMC and α-Toc in location and mobility. Previous studies suggest that the chromanol moiety of α-Toc is located in the hydrophobic region near the membrane surface and that the mobility of chromanol is restricted by the hydropho-

bic side chain, while PMC can move more freely within the membrane (10–13). Our results show that PCh is less active as a chain-breaking antioxidant in phospholipid membranes, regardless of the site of the chain initiation reaction. When a radical generator is used for initiating lipid peroxidation in membranes, α-Toc can scavenge chain-initiating peroxyl radicals as well as chain-propagating lipid peroxyl radicals. We presume that the chromanol moiety of PCh is located on the membrane surface and that the phospholipid moiety is inserted into the phospholipid bilayer, thereby restricting the mobility of PCh within the hydrophobic bilayer region. This location of PCh in membranes would lower the reactivity with chain-propagating lipid peroxyl radicals. The lateral diffusion rate of phospholipids in bilayers was reported to be about 270 times lower than that of α-Toc (32,33). Therefore, the lateral diffusion of the chromanol group of PCh would also be likely to be restricted within phospholipid bilayers resulting in the apparent lower activity with scavenging lipid peroxyl radicals.

We found that PMC is also less effective than α-Toc in preventing free radical initiated lipid peroxidation in the membrane model (Figs. 4 and 5) in spite of its ability to move freely within the bilayer. In addition, PMC and PCh were consumed more rapidly than α-Toc. α-Toc donates a hydrogen atom to a peroxyl radical to form an α-tocopheroxyl radical (8). Subsequent reaction of the d-α-tocopheroxyl radical with a second peroxyl radical gives both a stable product and an unstable product that may produce an alkoxyl radical (34-36). The lower effectiveness of PMC and PCh could be explained by the assumption that the reaction that produces the unstable products occurs quite easily in the case of PMC and PCh. Among the three compounds, α-Toc acts most efficiently as chain-breaking antioxidant in phospholipid bilayers. This supports the idea that the phytyl side chain of α-Toc arranges the chromanol moiety at a site suitable for scavenging chain-carrying lipid peroxyl radicals in biological membranes (7,10).

On the other hand, when the liposomal peroxidation was initiated in the presence of ascorbic acid, the antioxidant activity of PCh and PMC was the same as that of α-Toc (Fig. 6). Although ascorbic acid cannot inhibit lipid peroxidation within liposomes directly, it is known that it acts in the aqueous phase as a synergist by regenerating α-Toc (37,38). Bowry *et al.* (39) pointed out that the regeneration of α-Toc by ascorbic acid is required to prevent the prooxidant effect of α-Toc. We also found that ascorbic acid prolonged the induction period of α-Toc and its analogues in liposomal suspensions. It should also be emphasized that there is no difference in the antioxidant activity between α-Toc and PCh, although PCh is less effective in the absence of ascorbic acid. It appears that the regeneration by ascorbic acid is quite important in determining antioxidant activity in phospholipid bilayers when ascorbic acid is present in the aqueous phase.

In conclusion, we synthesized a new vitamin E phospholipid with antioxidant activity, which works as a chain-breaking antioxidant in phospholipid bilayers. Its activity is comparable to that of vitamin E in the presence of ascorbic acid, but its activity is lower in the absence of ascorbic acid. The new vitamin E phospholipid should be active in biological systems, provided the chromanol moiety is regenerated by ascorbic acid.

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Butylated Hydroxyanisole Inhibits Tumor Necrosis Factor-Induced Cytotoxicity and Arachidonic Acid Release

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The mechanisms by which the antioxidant butylated hydroxyanisole (BHA) inhibits recombinant tumor necrosis factor alpha (rTNF-α)-induced cytotoxicity have been studied in WEHI 164 clone 13 (WEHI) and L929 fibrosarcoma cells. When BHA was added simultaneously with rTNF-α, it completely inhibited rTNF-α cytotoxicity in the WEHI and L929 cells. BHA also inhibited the toxicity when added 2 h after rTNF-α in WEHI cells, suggesting that BHA inhibits some late intracellular event(s) in rTNF-α cytotoxicity. Pretreating WEHI cells with BHA for 4 h did not decrease the binding of rTNF-α to its receptors as measured using flow cytometry. BHA inhibited rTNF-α toxicity in the presence of actinomycin D and cycloheximide, indicating that neither mRNA nor protein synthesis is necessary for the BHA effect. The antioxidant butylated hydroxytoluene (BHT) and indomethacin did not inhibit the rTNF-α–induced cytotoxicity nor the rTNF-α-induced release of [^{3}H]arachidonic acid. By comparison, BHA completely inhibited the rTNF-α-induced release of arachidonic acid, suggesting that BHA somehow inhibits rTNF-α-induced activation of phospholipase(s). In WEHI cells, rTNF-α increased the level of protein-associated thiobarbituric acid reactive substances (TBARS) dose-dependently. BHA, but not BHT, blocked rTNF-α-induced cytotoxicity and rTNF-α-induced accumulation of protein-associated TBARS, suggesting that rTNF-α cytotoxicity is correlated with protein-associated TBARS. In conclusion, the results suggest that BHA blocks some post receptor event in rTNF-α-induced cytotoxicity, and that activation of phospholipase(s) coupled with the enzymatic formation of specific oxidized lipids could be a pivotal event in rTNF-α-induced cytotoxicity.
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Tumor necrosis factor (TNF) is a potent mononuclear macrophage derived cytokine with a variety of effects including the ability to enhance the growth of fibroblasts (1), to stimulate superoxide anion production by neutrophils (2) and to kill certain sensitive cancer cells (3,4).

Although the effects of TNF have been intensively studied both *in vivo* and *in vitro*, the biochemical events involved in TNF-induced cell killing are still not known in detail. TNF binds to cell membrane receptors (5,6), and two different receptors with different molecular weight have been identified (7). Heller *et al.* (8) recently reported that one of these receptors probably mediates TNF-induced cytotoxicity in murine TA1 cells. Following binding of TNF to its receptors, TNF activates several signal transduction pathways, including phosphatidylcholine specific phospholipase C (9), protein kinase C (9) and phosphorylation of intracellular proteins (10). Protein kinase C activity is probably not required for the TNF-induced activation of the transcription factor NF_KB, at least not in Jurkat cells (11). TNF also activates the sphingomyelin signal transduction pathway in a cell free system (12). In addition, a recent report suggested that TNF stimulates protein phosphatases (13). Clark *et al.* (14) showed that TNF stimulates phospholipase A_2 activity through induction of a phospholipase-activating protein in endothelial cells. In osteoblast-like cells, TNF stimulates phospholipase A_2 through receptor-mediated activation of G-proteins (15). Both TNF-induced cytotoxicity (16,17) and TNF-induced growth enhancement in FS-4 fibroblasts (17) are associated with enhanced release of arachidonic acid. However, which of these events are essential for TNF-induced cytotoxicity has not been clarified in detail. The intracellular calcium level does not change, at least not immediately after the addition of TNF to U937 cells (9). Several reports indicate that cyclooxygenase and lipoxygenase inhibitors (16,18), some antioxidants (18), dexamethasone (19) and the phospholipase A_2 inhibitor quinacrine (20), decrease TNF-induced cytotoxicity *in vitro*. TNF-induced cytotoxicity is associated with oxidative damage measured as increased levels of oxidized glutathione in some cell lines (21). Yamauchi *et al.* (22) showed that hydroxyl radicals are formed during TNF-induced cytotoxicity. Matthews *et al.* (18) reported that TNF induces accumulation of peroxides measured as thiobarbituric acid reactive substances (TBARS) in L929 cells sensitive to TNF-induced cytotoxicity, but not in TNF-resistant sublines.

We have recently shown that the antioxidant butylated hydroxyanisole (BHA) completely blocks the rTNF-α and rTNF-ß (lymphotoxin) toxicity in WEHI 164 clone 13 and L929 fibrosarcoma cells, while the structurally similar antioxidant butylated hydroxytoluene (BHT) had only little effect (23,24). BHA also inhibited rTNF-α-induced growth enhancement, but not interleukin-1ß-induced growth enhancement in FS-4 fibroblasts (24). Since TNF-induced cytotoxicity probably induces oxidative damage (18,21,22), the antioxidants BHA and BHT may be useful tools to study mechanism(s) involved in TNF-induced cytotoxicity. The present study therefore examined the mechanisms by which BHA inhibits rTNF-α-induced cytotoxicity in

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Abbreviations: ANOVA, analysis of variance; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; ED_{50}, effective dose reducing cell density to 50% of control; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FCS-M, 10% (vol/vol) heat-inactivated fetal calf serum in RPMI-1640 medium containing 2 mmol/L L-glutamine and 40 mg/L gentamicin sulfate; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PBS, phosphate buffered saline; rTNF-α, recombinant tumor necrosis factor alpha; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; UV, ultraviolet.

WEHI 164 and L929 fibrosarcoma cells. The effects of BHA and BHT on rTNF-α-induced cytotoxicity, accumulation of TBARS and release of arachidonic acid are reported.

MATERIALS AND METHODS

Materials. Fetal calf serum (FCS), L-glutamine, Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, trypsin solution and RPMI-1640 were obtained from Gibco Laboratories (Paisley, Scotland). RPMI-1640 without leucine and phenolic red was prepared from the RPMI-1640 select-amine kit (Gibco) and was sterilized by filtration through a 0.22 μm Sterivex-GS filter obtained from Millipore Corp. (Bedford, MA). Actinomycin D, arachidonic acid, bovine serum albumin (BSA), cycloheximide, *dl*-α-tocopherol, ethylenediaminetetraacetic acid (EDTA), indomethacin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quinacrine, sodium dodecylsulfate, 1,1,3,3-tetraethoxypropan, 2-thiobarbituric acid, trypan blue and BHT were obtained from Sigma Chemical Co. (St. Louis, MO). Gentamicin sulfate was obtained from Schering Corp. (Kenilworth, NJ), and ethanol was obtained from Vinmonopolet A/S (Oslo, Norway). BHA (mixture of 2-BHA and 3-BHA) and 4-hydroxymethyl-2,6-di-*t*-butylphenol was part of the food additive kit-92 from Supelco (Bellefonte, PA). Hydroxyeicosatetraenoic acids (HETE), prostaglandins and epoxy fatty acid standards for high-performance liquid chromatography (HPLC) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Citric acid monohydrate, trichloroacetic acid (TCA), hydrochloric acid, ethyl acetate of Uvasol quality, 1-butanol of pro analysis quality and methanol of Lichrosolv quality were obtained from E. Merck (Darmstadt, Germany). *n*-Hexane of HPLC quality was obtained from Fisons (FSA Laboratory Supplies, Loughborough, England). Propan-2-ol and acetonitrile (HPLC grade) were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland), and acetic acid of HPLC grade was obtained from Pierce Chemical Co. (Rockford, IL). The octadecyl (C_{18}) columns (500 mg) were purchased from Bond Elut (Varian SPP., Harbor City, CA). Murine rTNF-α with a specific activity of 8×10^7 U/mg protein, and human rTNF-α with a specific activity of 7.6×10^7 U/mg protein, as determined in a bioassay, were kindly provided by Genentech Inc. (South San Francisco, CA). Murine rTNF-α was used if not otherwise indicated. Streptavidin-phycoerythin was purchased from Becton-Dickinson & Co. (Mountain View, CA). L-[4,5-^{3}H]leucine (135 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (219 Ci/mmol) and [5,6,8,11,12,14,15(n)-^{3}H]prostaglandin E_2 (140 Ci/mmol) were obtained from Amersham International (Buckinghamshire, United Kingdom). Optifluor® scintillation fluid was purchased from Packard Instrument Company, Inc. (Downer's Grove, IL).

Cell culture. The WEHI 164 clone 13 cell line (termed WEHI clone 13 cells) which is highly sensitive to rTNF-α-induced cytotoxicity, was previously isolated from the WEHI 164 parental cell line (25). The L929 cell line was a generous gift from Dr. D. Løvhaug (Nycomed Bioreg, Oslo, Norway). WEHI and L929 cells were cultured in 75-cm^2 flasks (Costar 3275, Cambridge, MA) in RPMI-1640 containing 2 mM L-glutamine and 10% (vol/vol) heat-inactivated (56°C, 30 min) fetal calf serum (FCS-M). Cells were passed twice a week using trypsin and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Antioxidants were added to the FCS-M dissolved in ethanol. Ethanol at a final concentration of 0.05% (vol/vol) had no effect on rTNF-α-induced cytotoxicity.

Chromium release assay. rTNF-α-induced cytotoxicity was measured using the chromium release assay and was performed as previously described (23). The percent specific cell lysis was calculated as $100 \times (E\text{-}S)/(T\text{-}S)$, where E represents experimental [^{51}Cr]-release, S represents spontaneous release and T total release after lysing the cells with sodium dodecylsulfate at a final concentration of 0.5% (wt/vol).

Cell survival assay. Cell survival after exposure to rTNF-α was measured using the MTT assay as described (24). The stock solution contained MTT (5 g/L) dissolved in Dulbecco's PBS (without calcium and magnesium). WEHI clone 13 or L929 cells were trypsinized and seeded at 2×10^3 cells per well in microtiter plates (Costar 3598) using 200 μL FCS-M/well. The medium was removed after 48 h culture if not otherwise indicated. After 22 h incubation at 37°C with murine rTNF-α and the indicated inhibitors, the medium was again removed and 110 μL/well of FCS-M containing MTT at a final concentration of 0.45 g/L were added. The cultures were incubated for 4 h at 37°C, 50 μL of the supernatant was removed and 100 μL propan-2-ol containing 0.04 N HCl was added to dissolve the formazan. After the formazan had been dissolved, the optical density (OD) was measured on a Titertek Multiskan MCC/340 MK II microplate reader (Flow Lab, Irvine, Scotland) as described (24). Percent cell survival was calculated as: 100 × (OD in wells with TNF)/OD in wells without TNF).

rTNF-α receptor binding. WEHI clone 13 cells were seeded in 1400-mm microbiological petri dishes (Nunc A/S, Roskilde, Denmark) at 2×10^6 cells/dish, and cultured as nonadherent cultures in 15 mL FCS-M. After 22–24 h incubation, 5 mL FCS-M containing BHA or BHT (100 μM) was added, and the incubation continued for 4 h. Cells were pelleted at +4°C, FCS-M added (+4°C), and kept at 0–4°C. Thereafter, 10^6 cells were treated with biotinylated human rTNF-α in PBS containing BSA (PBS/BSA) for 45 min, washed twice in PBS/BSA and stained with streptavidin–phycoerythrin for 45 min at 0–4°C. Cells were subsequently washed in PBS, fixed in 2% (vol/vol) formalin and stored at 0–4°C. Thereafter, 5×10^3 cells were analyzed on a FACScan flow cytometer (Becton Dickinson & Co.) as previously described (6).

Protein synthesis assay. Protein synthesis was measured essentially as described by Sandvig *et al.* (26). WEHI clone 13 cells were seeded at a density of 2×10^3 cells/well in microplates using 200 μL FCS-M/well, and the incubation continued for 44 h. Protein synthesis was measured by adding [^{3}H]leucine (1 μCi/well) in 10 μL

RPMI-1640 without leucine and phenolic red, and continuing the incubation for 30 min. The medium was carefully removed and cell proteins precipitated and washed once with 5% (wt/vol) TCA (26). Proteins were dissolved in 40 µL KOH (0.1 M), samples mixed with 10 mL Optifluor^R scintillation fluid and radioactivity determined using an LKB Wallac 1211 Rackbeta counter (Turku, Finland).

TBARS assay. WEHI clone 13 or L929 cells were trypsinized and seeded in 1400-mm microbiological petri dishes at 2×10^6 cells/dish in 10 mL FCS-M. After 4 h of incubation, control medium or medium (10 mL) containing unlabeled arachidonic acid was added as indicated. After approximately 47 h of total incubation, the cells preincubated with arachidonic acid were resuspended, centrifuged and seeded in microbiological petri dishes at 1×10^6 cells/mL in FCS-M. Cells received rTNF-α in the absence or presence of antioxidant after approximately 48 h of total incubation. The cells were resuspended 22 h later and aliquots taken for cell counting and determination of viability by trypan blue exclusion. The cells were counted using a Coulter Counter ZF (Coulter Electronics, Dunstable Beds, England) or a Bürker counting chamber. Cells were pelleted at +4°C ($800 \times g$, 15 min), washed twice in 0.9% (wt/vol) NaCl at +4°C, and finally resuspended in 0.8 mL NaCl (+4°C). Aliquots were taken for protein analysis using the Coomassie brilliant blue technique (27) with BSA as standard. Thereafter, cells were lysed and proteins precipitated by adding 10% (wt/vol) TCA. TBARS were measured according to Chow *et al.* (28), using reduced reagent volumes to increase sensitivity. When TBARS in non-TCA precipitable material were measured, samples were centrifuged after precipitation of cell proteins and the supernatant moved to a new tube before addition of thiobarbituric acid. Thereafter, TBARS were extracted with 1-butanol and analyzed in cells from two wells to further increase sensitivity of the assay. 1,1,3,3-Tetraethoxypropane from 1 to 10 µM was used as standard. Absorbance at 532 nm was measured on a Perkin Elmer Lambda 5 spectrophotometer (Norwalk, CT), or on the Titertek Multiskan microplate reader in the dual wavelength mode with test and reference wavelengths set at 540 and 620 nm, respectively. The characteristic peak at 532 nm in the ultraviolet (UV)-spectrum was measured in test samples and standards using the Perkin Elmer Lambda 5 spectrophotometer.

Release of [3H]arachidonic acid. The effect of BHA and BHT on rTNF-α-induced release of arachidonic acid from L929 cells was investigated in cells prelabeled with [^{3}H]arachidonic acid. L929 cells were seeded at 0.5×106 cells in 60-mm petri dishes (Costar 3060). Approximately 24 h after seeding, cells received RPMI-1640 containing 1% (vol/vol) FCS, 2 mM L-glutamine and [^{3}H]arachidonic acid (1 mCi/L) in a total volume of 2 mL. After 24 h of further incubation, the culture medium was removed and cells were washed three times with FCS-M (37°C) to remove extracellular [^{3}H]arachidonic acid. Thereafter, rTNF-α and antioxidants were added as indicated in 2 mL FCS-M. FCS-M containing RPMI-1640 without phenolic red was used to avoid quenching during liquid scintillation counting. The culture medium was removed at the indicated time points after the addition of rTNF-α and centrifuged ($500 \times g$, 10 min) to remove loose cells. Thereafter, aliquots (100 µL) of the supernatants were mixed with 10 mL Optifluor^R scintillation fluid and radioactivity measured by liquid scintillation counting on the LKB Wallac 1211 Rackbeta counter. In some experiments, the culture medium was removed after 4 h of incubation with rTNF-α and the adherent cells detached by adding 1 mL trypsin containing 0.2 g/L of EDTA (37°C). After detachment of the cells, the trypsin-EDTA solution was immediately neutralized by addition of 1 mL FCS-M (+4°C). The culture medium was centrifuged at +4°C ($500 \times g$, 10 min), and the pellet containing nonadherent cells was combined with the trypsinized cells before determination of viability using trypan blue exclusion.

Viability. Cell viability was determined by dye exclusion using trypan blue at a final concentration of 0.2% (wt/vol). The samples were kept at +4°C, and 100 cells were counted two or three times in each sample using a hemocytometer. The release of lactate dehydrogenase to the medium was measured using a standard UV method.

Extraction and HPLC of arachidonic acid metabolites. Arachidonic acid metabolites were extracted according to Powell (29) and analyzed by HPLC as previously described (24). Recovery of [^{3}H]arachidonic acid and [^{3}H]prostaglandin E_2 added to FCS-M was 97 ± 4% and 88 ± 4% (mean ± SD, n = 3), respectively. The samples were analyzed by HPLC using a 5 µm Supelcosil LC-18 (150 mm × 4.6 mm i.d.) reversed-phase column. The HPLC method used in this report measured prostaglandins, HETE and free fatty acids, but did not measure peptido-leukotrienes (29). Metabolites were identified by coelution with standards in two different HPLC gradients.

Statistics. The data were compared by one-way analysis of variance (ANOVA) using the SPSS-PC+ version 4.01 statistical program obtained from SPSS Inc. (Chicago, IL).

RESULTS

Effect of the antioxidant BHA on rTNF-α-induced cytotoxicity. Addition of BHA simultaneously with rTNF-α completely inhibited cytotoxicity both in WEHI clone 13 (Fig. 1A) and L929 cells (Fig. 1B). To examine whether BHA inhibited early or late events in rTNF-α-induced cytotoxicity, we also added BHA at different time points after rTNF-α addition (Fig. 1). Adding BHA 2 or 4 h after rTNF-α gave approximately the same protection as when BHA was added simultaneously with rTNF-α (Fig. 1). When BHA was added 8 h after rTNF-α in the L929 cells, cell death decreased from 37% in the control to 21% when BHA was added (Fig. 1B). However, since 20% of the cells were already dead at the time when BHA was added, the protection of the remaining viable cells was still approximately 94%. Similar results were found in the WEHI cells (Fig. 1A). This indicates that BHA inhibits rTNF-α-induced cytotoxicity as late as

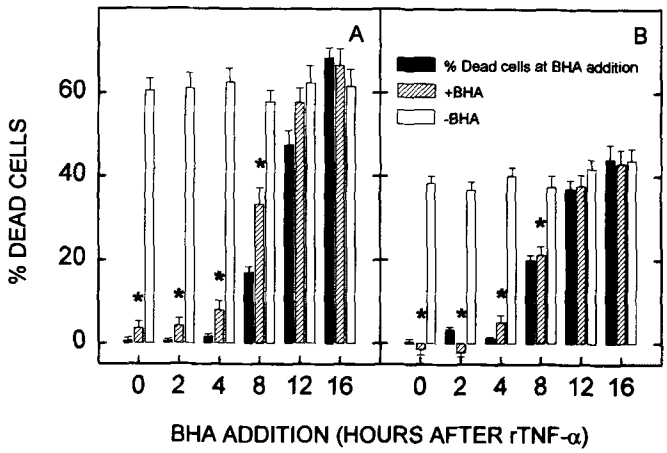

FIG. 1. The effect of delayed addition of butylated hydroxyanisole (BHA) on recombinant tumor necrosis factor α (rTNF-α)-induced cytotoxicity in (A) WEHI clone 13 cells and (B) L929 cells. All incubations were started by adding 40 ng/L (WEHI clone 13) or 160 ng/L of rTNF-α (L929 cells). Explanation of symbols: filled bar: no additions, percent dead cells at the indicated times; hatched bar: medium containing 100 μM BHA added at the indicated times, and percent dead cells measured 16 h after the addition of rTNF-α. Open bar: control medium without BHA added at the indicated times, and percent dead cells measured 16 h after the addition of rTNF-α. Cell death was measured using the [^{51}Cr]-release assay. Spontaneous release of [^{51}Cr] after 16 h incubation was 20 and 24% of total in WEHI and L929 cells, respectively. The results are from one of two similar experiments (WEHI) and one experiment (L929), and are given as means ± SD of six parallels. *BHA significantly different ($P < 0.05$) from control medium when analyzed by one-way analysis of variance using Duncan multiple comparison test.

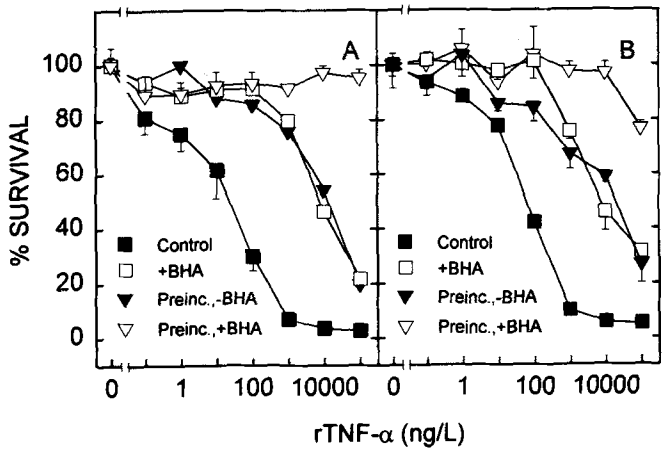

FIG. 2. The effect of BHA after 2-h pretreatment with rTNF-α in (A) WEHI clone 13 cells and (B) L929 cells. Cells were seeded in microtiter plates, and further incubated for 48 h. Thereafter, the medium was removed, and the cells were incubated 22 h with the indicated concentrations of rTNF-α without BHA (■), or in the presence of 100 μM BHA (□). In some wells, rTNF-α was removed after 2 h of incubation, and the cells washed three times with complete medium before continuing the incubations in control medium (▼), or medium containing 100 μM of BHA (▽). Cell survival was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after a total of 22 h of incubation. The results are from one of two similar experiments, and are presented as mean ± SD of triplicates. See Figure 1 for abbreviations.

6–8 h after the binding of rTNF-α to its receptors, suggesting that BHA probably inhibits some late event(s) in rTNF-α-induced cytotoxicity.

We next examined whether BHA mediates its effect by binding or inactivating rTNF-α in the culture medium. Cells were preincubated for 2 h with rTNF-α and any remaining extracellular rTNF-α was removed by washing the cells extensively before adding BHA (Fig. 2). BHA inhibited the toxicity also when extracellular rTNF-α had been removed by washing, indicating that BHA does not act by binding or inactivating rTNF-α in the culture medium.

We thereafter tested whether BHA down-regulated rTNF-α receptor binding. Preincubation with BHA or BHT did not decrease the binding of human rTNF-α to its receptors in WEHI clone 13 cells as measured using flow cytometry. This suggests that BHA probably affects some post receptor event in rTNF-α-induced cytotoxicity. Since we used human rTNF-α in the receptor binding studies, we verified that BHA and BHT (100 μM) affected human rTNF-α toxicity similarly as murine rTNF-α toxicity (data not shown).

Effect of preincubation with BHA. Since others have reported that BHA induces the activity of several enzymes, including the glutathione *S*-transferase (30), we examined whether preincubation with BHA protected against rTNF-α cytotoxicity. Preincubation with BHA for 24 or 48 h followed by removal of BHA neither had any effect on rTNF-α cytotoxicity, nor on BHA protection against rTNF-α cytotoxicity (Table 1). This indicates that BHA probably does not mediate its effect by inducing enzyme synthesis, or by activating some unknown defense mechanism to give a lasting protection against rTNF-α-induced cytotoxicity.

BHA protection in the presence of actinomycin D or cycloheximide. We next examined whether the inhibitory effect of BHA on rTNF-α cytotoxicity depends on mRNA or protein synthesis. BHA protected completely against rTNF-α cytotoxicity in the presence of the mRNA synthetase inhibitor actinomycin D at rTNF-α concentrations up to 1 ng/L, corresponding to 25% death in the control cells using [^{51}Cr]-release (Fig. 3A). BHA protected only partially at higher rTNF-α concentrations, possibly because actinomycin D potentiated rTNF-α toxicity. Similarly, BHA completely inhibited rTNF-α toxicity in the presence of the protein synthesis inhibitor cycloheximide up to 10 ng/L of rTNF-α (Fig. 3B). This suggests that BHA does not depend on protein synthesis to protect against rTNF-α cytotoxicity. However, since actinomycin D and cycloheximide were added simultaneously with rTNF-α, we could not exclude that BHA stimulated protein synthesis before it was inhibited by actinomycin D or cycloheximide.

In the next experiment, cells were preincubated for 4 h with actinomycin D or cycloheximide to ensure that protein synthesis was inhibited when rTNF-α and BHA were added (Fig. 4). BHA again inhibited rTNF-α cytotoxicity

TABLE 1

Effect of Preincubation with BHA on rTNF-α-Induced Cytotoxicity in WEHI Clone 13 Cells

	Optical density[a]			
	24-h Preincubation		48-h Preincubation	
	-BHA	+BHA	-BHA	+BHA
Incubation[b]				
Control	0.78 ± 0.10	0.75 ± 0.12	0.83 ± 0.11	0.75 ± 0.11
rTNF-α	0.25 ± 0.07[c]	0.26 ± 0.07[c]	0.21 ± 0.08[c]	0.24 ± 0.07[c]
BHA	0.77 ± 0.17	0.74 ± 0.16	0.81 ± 0.18	0.76 ± 0.18
rTNF-α + BHA	0.75 ± 0.08	0.68 ± 0.10	0.74 ± 0.06[c]	0.70 ± 0.09

[a]WEHI clone 13 cells were seeded at 2×10^3 cells/well in microplates in 200 µL 10% (vol/vol) heat-inactivated fetal calf serum in RPMI-1640 medium containing 2 mmol/L L-glutamine and 40 mg/L gentamicin sulfate (FCS-M). All cells were preincubated for 48 h, and butylated hydroxyanisole (BHA) (100 µM) was present during the last 24 or 48 h of preincubation as indicated.

[b]Additions during incubation. After preincubation, the medium was removed, and the cells received either control medium, recombinant tumor necrosis factor alpha (rTNF-α) (1 µg/L), BHA (100 µM) or rTNF-α plus BHA. Cell density was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods, and the results are expressed as optical density. The results are from five experiments, each performed in quadruplicate, and are given as means ± SD of 20 determinations.

[c]Statistically different ($P < 0.05$) from the control when each preincubation was analyzed separately by one-way analysis of variance using Duncan multiple comparison test.

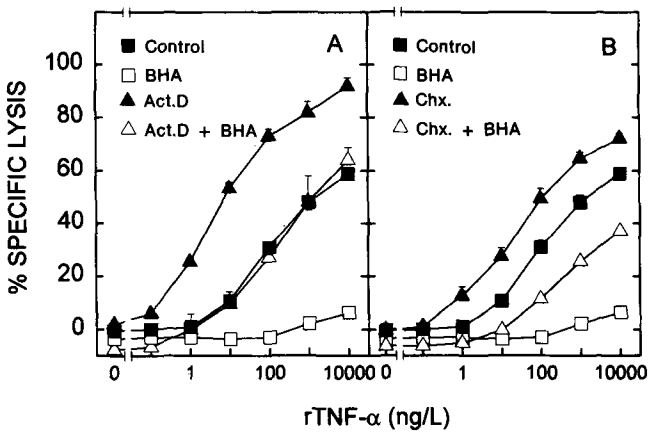

FIG. 3. The effect of BHA on rTNF-α cytotoxicity in L929 cells in the presence of (A) actinomycin D (Act.D) or (B) cycloheximide (Chx.) Cells received Act.D (2 mg/L) or Chx. (2 mg/L) simultaneously with rTNF-α in the presence or absence of BHA (100 µM), and cytotoxicity was measured 16 h later using the [^{51}Cr]-release assay as described in Materials and Methods. Results are expressed as percentage specific lysis and given as means ± SD of triplicates. The spontaneous release after 16 h of incubation was 23% of total release. Additions: A: (■) none; (□) 100 µM BHA; (▲) 2 mg/L of Act.D; (△) 2 mg/L of Act.D and 100 µM BHA; B: (■) none; (□) 100 µM BHA; (▲) 2 mg/L of Chx.; (△) 2 mg/L of Chx. and 100 µM BHA. See Figure 1 for other abbreviations.

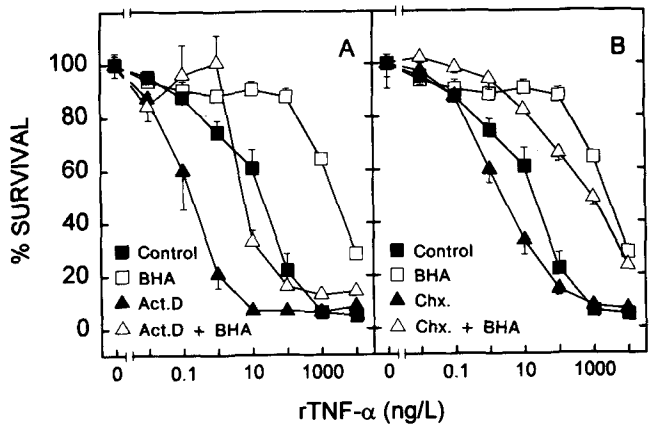

FIG. 4. The effect of BHA on rTNF-α-induced cytotoxicity in WEHI clone 13 cells preincubated 4 h in the absence or presence of (A) 1 mg/L of Act.D and (B) 1 mg/L of Chx. After 44 h of incubation, the medium was removed and control medium, Act.D (1 mg/L) or Chx. (1 mg/L) added in 100 µL 10% (vol/vol) heat-inactivated fetal calf serum in RPMI-1640 medium containing 2 mmol/L L-glutamine and 40 mg/L gentamicin sulfate (FCS-M) and after 4 h further incubation, rTNF-α was added in tenfold dilutions in the presence or absence of BHA in a total volume of 200 µL FCS-M/well. The percentage cell survival was measured after 22 h further incubation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results are from one of four similar experiments, and are given as means ± SD of quadruplicates. Additions: A: (■) none; (□) 250 µM BHA; (▲) 1 mg/L Act.D; (△) 1 mg/L of Act.D and 250 µM of BHA; B: (■) none; (□) 250 µM BHA; (▲) 1 mg/L Chx.; (△) 1 mg/L of Chx. and 250 µM of BHA, and the mean optical density in the absence of rTNF-α was: 0.90, 0.86, 0.38, 0.34, 0.90, 0.86, 0.43 and 0.38, respectively. See Figures 1 and 3 for other abbreviations.

almost completely at rTNF-α concentrations up to 1 ng/L in the presence of actinomycin D (Fig. 4A), while BHA protection became negligible at rTNF-α concentrations above 100 ng/L in WEHI cells. BHA increased the effective dose

TABLE 2

Effect of Actinomycin D and Cycloheximide on Protein Synthesis in WEHI Cells[a]

	Incorporation of [^{3}H]leucine (percent of control)					
	4 h[b]			4 + 4 h[b]		
Addition[c]	Control	+ Chx.	+ Act.D	Control	+ Chx.	+ Act.D
None	100 ± 13.7	13 ± 3.1[d]	70 ± 10.7[d]	100 ± 7.9	10 ± 2.4[d]	48 ± 10.6[d]
rTNF-α	94 ± 30.2	12 ± 2.4[d]	66 ± 8.4[d]	88 ± 15.4[d]	9 ± 2.0[d]	22 ± 5.8[d,e]
BHA	82 ± 19.4[d]	9 ± 0.9[d]	69 ± 6.0[d]	84 ± 23.5[d]	11 ± 1.9[d]	50 ± 5.7[d]
rTNF-α + BHA	70 ± 10.7[d]	8 ± 2.5[d]	54 ± 14.8[d,e]	72 ± 23.5[d]	7 ± 1.9[d]	29 ± 12.9[d,e]

[a]WEHI clone 13 cells were seeded at 2×10^3 cells/well in microplates using 200 µL FCS-M/well and the medium was changed after 44 h of incubation. See Table 1 for other abbreviations.

[b]Protein synthesis was measured after 4 h of preincubation with control medium, cycloheximide (Chx., 1 mg/L) or actinomycin D (Act.D, 1 mg/L) as indicated, or after 4 h of preincubation followed by 4 h of incubation with the indicated additions.

[c]Additions during incubation and during assay of protein synthesis. After 4 h of preincubation, cells were incubated further for 4 h with control medium, rTNF-α (1 µg/L), BHA (250 µM) or rTNF-α plus BHA in the presence or absence of Chx. or Act.D as indicated. Protein synthesis was measured by adding [^{3}H]leucine (1 µCi/well) as described in Materials and Methods. The results are calculated as percent of mean cpm in controls (14783 cpm and 21522 cpm after 4 h of preincubation and 4 + 4 h of incubation, respectively). The results are from three experiments, each performed in triplicate and are given as means ± SD of data from nine cultures.

[d]Significantly different ($P < 0.05$) from the control with no addition when analyzed by one-way analysis of variance (ANOVA) using Duncan multiple comparison test. Each time point was analyzed separately.

[e]Significantly different ($P < 0.05$) from Chx. with no addition or Act.D with no addition, when analyzed by one-way ANOVA using Duncan multiple comparison test. Each time point was analyzed separately.

reducing cell density to 50% of control (ED_{50}) of rTNF-α approximately 180-fold in the absence, and approximately 75-fold in the presence of actinomycin D. BHA also inhibited rTNF-α-induced cytotoxicity after 4 h preincubation with the protein synthesis inhibitor cycloheximide (Fig. 4B). At an rTNF-α concentration giving 30% survival, BHA increased cell survival to approximately 80% in the presence and 90% in the absence of cycloheximide. Preincubation with cycloheximide or actinomycin D for 4 h reduced protein synthesis measured as incorporation of [^{3}H]leucine to 13 and 70% of control, respectively (Table 2). BHA alone (250 µM) inhibited protein synthesis only slightly. The results also confirmed that actinomycin D and cycloheximide inhibited protein synthesis also during incubation with rTNF-α and/or BHA (Table 2).

Effect of BHA on rTNF-α-induced TBARS. We next examined whether BHA inhibited the rTNF-α-induced peroxidation measured as TBARS. rTNF-α dose-dependently increased both the percentage of dead cells and of protein-associated TBARS (Fig. 5A). However, there was no linear relationship between TBARS accumulation and cell death. BHA (100 µM) reduced rTNF-α toxicity from 85 to 13% dead cells, respectively, and at the same time decreased TBARS from 517 to 126% of controls (Fig. 5B). rTNF-α increased TBARS also when TBARS were calculated as pmol/culture well or as pmol/10^6 cells. We did not detect TBARS in the culture medium (results not shown). In a control experiment, WEHI cells treated with water contained 100% dead cells and 134 pmol TBARS/mg protein compared to control cells which contained only 5% dead cells and 138 pmol TBARS/mg protein (mean, n = 3). This suggests that TBARS were not only a measure of cell death.

To examine whether the effect of BHA on TBARS accumulation and rTNF-α cytoxicity was due to its antioxidant effect, we compared BHA with the similar antioxidant

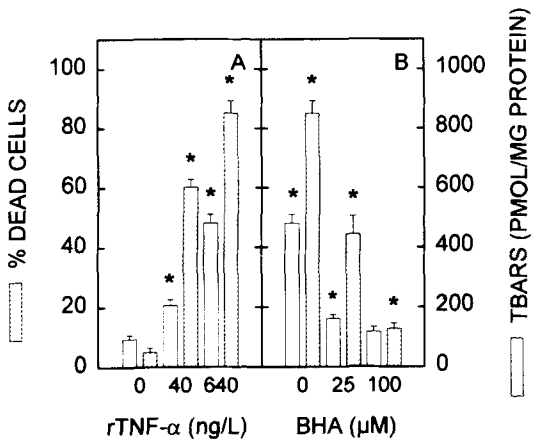

FIG. 5. The effect of BHA on rTNF-α-induced accumulation of protein-associated thiobarbituric acid reactive substances (TBARS) in WEHI clone 13 cells. The WEHI cells were incubated for 22 h with (A): 0, 40 or 640 ng/L of rTNF-α or (B): 640 ng/L of rTNF-α in the presence of 0 µM, 25 µM and 100 µM of BHA. Percentage of dead cells was measured by trypan blue exclusion, and TBARS were analyzed as described in Materials and Methods. Hatched bars: percentage of dead cells given as mean ± SD of six determinations; open bars: pmol TBARS per mg protein given as mean ± SD of triplicate cultures. One of two similar experiments is shown. *$P < 0.05$ as compared to the control when analyzed by one-way analysis of variance using Duncan multiple comparison test. See Figure 1 for other abbreviations.

BHT (Table 3). In WEHI clone 13 cells, rTNF-α gave 29% dead cells and increased protein-associated TBARS to 262% of the control without antioxidant. BHT neither inhibited rTNF-α cytotoxicity nor TBARS accumulation. In comparison, BHA blocked rTNF-a toxicity completely and at the same time reduced protein-associated TBARS to the control level. Similar results were also obtained using

TABLE 3

Effect of BHA and Butylated Hydroxytoluene (BHT) on rTNF-α-Induced TBARS Accumulation[a]

	−rTNF-α		+rTNF-α	
Additions (μM)	TBARS pmol/mg protein	Dead cells (%)	TBARS pmol/mg protein	Dead cells (%)
WEHI clone 13 cells				
None	87 ± 18	(3 ± 1)	228 ± 76[b]	(29 ± 5)[b]
BHA (100)	98 ± 18	(5 ± 2)	89 ± 7.2	(6 ± 1)
BHT (100)	89 ± 18	(4 ± 1)	271 ± 88[b]	(60 ± 6)[b]
L929 cells				
None	74 ± 15	(1 ± 1)	199 ± 34	(34 ± 3)
BHA (100)	85 ± 11	(2 ± 1)	90 ± 24	(2 ± 1)
BHT (100)	65 ± 11	(2 ± 1)	198 ± 3	(27 ± 2)

[a]WEHI clone 13 and L929 cells were cultured in 1400-mm petri dishes for 48 h as described in Materials and Methods. Thereafter, cells received 40 ng/L of rTNF-α (WEHI clone 13) or 160 ng/L (L929 cells) as indicated, and the incubation continued for 22 h. Thereafter, cell proteins were precipitated using trichloroacetic acid and protein-associated thiobarbituric acid reactive substances (TBARS) measured as described in Materials and Methods. The results are from two independent experiments in WEHI cells and are given as means ± SD of quadruplicate cultures. The results in L929 cells are from one experiment and are given as means ± SD of duplicates. Percent dead cells was measured using trypan blue exclusion, and the results are given as means ± SD of quadruplicate cultures (WEHI) or duplicate cultures (L929). See Table 1 for other abbreviations.

[b]Significantly different ($P < 0.05$) when compared to the WEHI cell control when analyzed by one-way analysis of variance using Duncan multiple comparison test.

L929 cells (Table 3). In a control experiment, BHT was added during the heating of the samples as described by Asakawa and Matsushita (31). Again BHA, but not BHT, inhibited rTNF-α-induced protein-associated TBARS, suggesting that TBARS were not due to peroxidation during the TBARS assay (data not shown). The results suggest that the protective effect of BHA on accumulation of protein-associated TBARS and rTNF-α cytotoxicity is probably not related to its antioxidant effect.

To test whether rTNF-α increases water-soluble TBARS, TBARS were measured in the supernatant after precipitation of the cell proteins with TCA. TBARS in non-TCA precipitable material were below the detection level in WEHI clone 13 cells not preincubated with arachidonic acid (data not shown). However, when WEHI cells were preincubated for 44 h with 50 μM arachidonic acid, rTNF-α induced the accumulation of TBARS in non-TCA precipitable material (Fig. 6). Both BHA and BHT similarly inhibited the rTNF-α-induced accumulation of TBARS in non-TCA precipitable material (Fig. 6). Since only BHA protected against rTNF-α cytotoxicity (Fig. 6), these TBARS are not associated with rTNF-α cytotoxicity. However, BHA, but not BHT, inhibited the rTNF-α-induced accumulation of protein-associated TBARS in WEHI cells enriched with arachidonic acid (data not shown), indicating again that protein-associated TBARS are associated with rTNF-α toxicity.

Differential effect of BHA and BHT on rTNF-α-induced release of [3H]arachidonic acid and prostaglandin E_2 synthesis. Since others have reported that TNF enhances the release of arachidonic acid in cells sensitive to TNF toxicity (32), we tested whether BHA and BHT affected the rTNF-α-induced release of [^{3}H]arachidonic acid. To avoid nonspecific release of [^{3}H]arachidonic acid secondary to cell lysis, we compared the effect of antioxidants after 4 h of incubation with rTNF-α. At this time, cell death had been induced (Fig. 2), but was not yet detectable (Table 4).

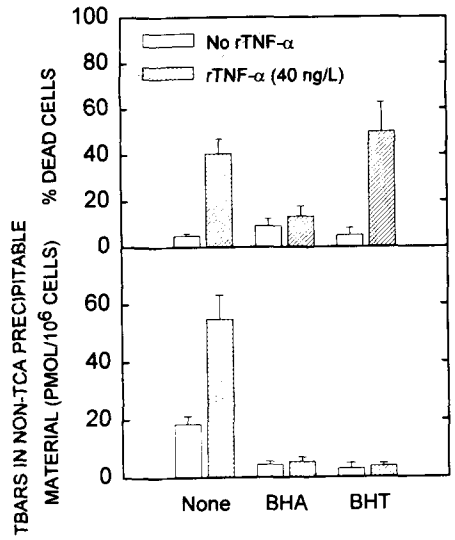

FIG. 6. The effect of BHA and butylated hydroxytoluene (BHT) on rTNF-α-induced formation of TBARS in non-trichloroacetic acid (TCA) precipitable material in WEHI clone 13 cells. The WEHI cells were seeded at 2×10^6 cells per dish, added 50 μM unlabeled arachidonic acid after 4-h culture, and the incubation continued for 44 h. Thereafter the medium was changed, and cells received control medium (open bar) or 40 ng/L of rTNF-α (hatched bar) in the presence of either 100 μM of BHA or BHT as indicated and the incubation continued for 22 h. TBARS in non-TCA precipitable material were measured in the supernatant after precipitation of cellular proteins with TCA as described in Materials and Methods. Upper panel: percentage of dead cells measured using trypan blue exclusion and given as mean ± SD of triplicates. Lower panel: TBARS in non-TCA precipitable material per 10^6 cells. Data are from three independent experiments and are given as mean ± SD of triplicates. See Figures 1 and 5 for other abbreviations.

rTNF-α enhanced the release of [^{3}H]arachidonic acid to 231% of control, and BHA inhibited this release dose dependently (Fig. 7A). BHT alone slightly increased the spontaneous release of [^{3}H]arachidonic acid, while it had

TABLE 4

The Influence of Antioxidants and Indomethacin on rTNF-α-Induced Release of [^{3}H]Arachidonic Acid in L929 Cells

Additions[a]	AA release (cpm/well)[b]	Dead cells (%)	LDH release (%)
Control	11503 ± 155[d]	3.1 ± 1.8	7.2 ± 0.2
BHA	11393 ± 372	2.1 ± 0.2	7.9 ± 0.3
HBP	10006 ± 186[d]	2.6 ± 1.3	7.2 ± 0.4
DL-α-tocopherol	11962 ± 330[d]	1.1 ± 0.2[d]	7.0 ± 0.3
Indomethacin	17626 ± 382[c,d]	2.3 ± 1.1	—[e]
rTNF-α	26706 ± 2084[c]	4.0 ± 1.7	7.5 ± 0.6
rTNF-α + BHA	12330 ± 561[d]	2.4 ± 2.0	7.2 ± 0.5
rTNF-α + HBP	12181 ± 886	2.9 ± 2.1	6.7 ± 1.2
rTNF-α + *dl*-α-tocopherol	23699 ± 744[c,d]	2.0 ± 0.1	8.5 ± 0.4
rTNF-α + indomethacin	29991 ± 1636[c]	2.1 ± 0.5	—[e]

[a]L929 cells were seeded at 0.5 × 10^6 cells/petri dish in 3 mL FCS-M. After approximately 24 h of incubation, the medium was changed to 1% (vol/vol) FCS-M containing 1 mCi/L of [^{3}H]arachidonic acid. After further 24 h incubation, the cells were washed three times with FCS-M and rTNF-α (1 μg/L) added simultaneously with 100 μM of BHA, 4-hydroxymethyl-2,6-di-*t*-butylphenol (HBP), *dl*-α-tocopherol or indomethacin, as indicated in 2 mL FCS-M containing RPMI without phenolic red. The supernatants were harvested after 4 h of incubation. Aliquots were counted in a liquid scintillation counter, and the results expressed as cpm/well and given as mean ± SD of duplicate cultures from one of three similar experiments. Percent dead cells was determined by trypan blue exclusion after trypsinization of the cells, and the results are pooled from two experiments and given as means ± SD of three or four incubations. Release of lactate dehydrogenase (LDH) is expressed as percent of total release in control cells lysed with Triton-X-100 and is given as means ± SD of duplicate incubations. See Table 1 for other abbreviations.

[b]Release of [3H]arachidonic acid (AA) into the cell culture medium.

[c]$P < 0.05$ compared to the control when analyzed by one-way analysis of variance (ANOVA) using Duncan multiple comparison test.

[d]$P < 0.05$ compared to rTNF-α when analyzed by one-way ANOVA using Duncan multiple comparison test.

[e]—, Not determined.

no effect on the rTNF-α-induced release (Fig. 7A). Separate time course experiments showed that the rTNF-α-enhanced release of [^{3}H]arachidonic acid started approximately 1–2 h after the addition of rTNF-α (Fig. 7B). This is approximately 2–3 h before the onset of cell death.

We next compared the effect of BHA on rTNF-α-induced release of [^{3}H]arachidonic acid with other antioxidants and indomethacin (Table 4). The antioxidant 4-hydroxymethyl-2,6-di-*t*-butylphenol, which completely blocks rTNF-α-induced toxicity (24), inhibited the rTNF-α-induced release of [^{3}H]arachidonic acid in L929 cells. By comparison, α-tocopherol or indomethacin neither inhibited rTNF-α-induced release of [^{3}H]arachidonic acid nor rTNF-α-induced cytotoxicity as previously shown (23,24). Indomethacin itself increased the release of [^{3}H]arachidonic acid into the culture medium. After 4 h, rTNF-α enhanced the release of [^{3}H]-arachidonic acid without any increase in dead cells, suggesting that this release was not secondary to cell death. In a separate experiment, the phospholipase inhibitor quinacrine (10 μM) inhibited the rTNF-α-induced release of [^{3}H]arachidonic acid by 70 ± 5.5% (mean ± SD, n = 2) after 4 h of incubation with rTNF-α, suggesting that rTNF-α induces phospholipase activity in L929 cells. Quinacrine also inhibited rTNF-α cytotoxicity by 59 ± 13% (mean ± SD, n = 3) as determined by trypan blue exclusion after 22 h of incubation with rTNF-α (1 μg/L). Addition of rTNF-α did not inhibit the incorporation of [^{3}H]arachidonic acid into L929 phospholipids (data not shown), indicating that the enhanced release of [^{3}H]arachidonic acid was not due to a reduced incorporation of the fatty acid.

Since the rTNF-α-induced release of [^{3}H]arachidonic acid was inhibited by BHA but not by BHT, we thereafter examined whether rTNF-α stimulated the release of arachidonic acid metabolites in L929 cells using HPLC analysis. Approximately 90% of the radiolabel released into the medium after 4 h of incubation with rTNF-α was arachidonic acid as measured using HPLC (data not shown). Addition of rTNF-α induced the synthesis of prostaglandin E2, which started approximately 4 h after the addition of rTNF-α (Table 5). Both BHA and indomethacin inhibited prostaglandin synthesis completely after 12 h of incubation with rTNF-α, while BHT had minimal effects. Since only BHA, but not BHT and indomethacin, inhibited rTNF-α-induced cytotoxicity both in WEHI clone 13 (23) and L929 cells (data not shown), the effect of BHA on rTNF-α toxicity cannot be explained through inhibition of prostaglandin E_2 synthesis only.

DISCUSSION

The present report shows that the antioxidant BHA inhibited rTNF-α-induced cytotoxicity in L929 and WEHI clone 13 fibrosarcoma cells, while BHT had no effect. The results suggest that BHA, but not BHT, inhibits rTNF-α-induced phospholipase activity, and that the effect is independent of protein or mRNA synthesis. BHA may inhibit rTNF-α-induced toxicity by at least three different mechanisms that will be discussed below.

TABLE 5

The Influence of BHA and BHT on rTNF-α-Induced-Prostaglandin E_2 Synthesis in L929 Cells

Additions[a]	Incubation time with rTNF-α	
	4 h	12 h
Control	ND[b]	ND[b]
BHA	ND	ND
BHT	ND	ND
Indomethacin	ND	ND
rTNF-α	12 (0–64)	294 (281–307)
rTNF-α + BHA	ND	ND
rTNF-α + BHT	ND	214 (198–230)
rTNF-α + indomethacin	ND	ND

[a]L929 cells were seeded in 60-mm petri dishes and cultured as described in Materials and Methods. The medium was removed and rTNF-α (1 μg/L) added simultaneously with 100 μM of BHA, BHT or indomethacin as indicated. The supernatants were harvested after 4 h or 12 h of incubation, and eicosanoids extracted and analyzed by high-performance liquid chromatography. The amount of prostaglandin E_2 is given as the relative areas on the radioactivity detector. The detection limit was approximately 150 cpm of [^{3}H]prostaglandin E_2, corresponding to a relative area of 29. The results are the means and ranges from 2–4 separate experiments. See Tables 1 and 3 for abbreviations.

[b]ND, *not detected.*

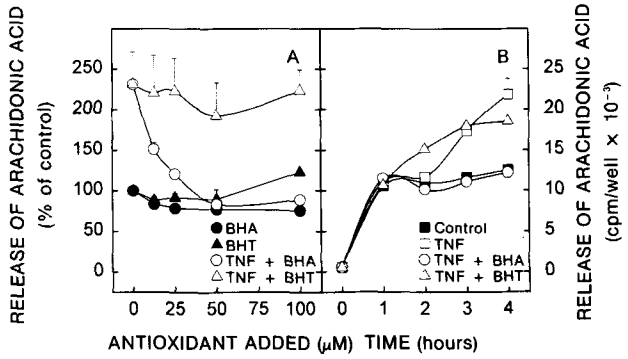

FIG. 7. The effect of BHA and BHT on rTNF-α-induced release of [^{3}H]arachidonic acid in L929 cells. A: Dose-dependent effect of BHA and BHT on the release of [^{3}H]arachidonic acid 4 h after the addition of rTNF-α. Cells were seeded in petri dishes and after 24 h culture, the medium was changed to 1% (vol/vol) fetal calf serum in RPMI-1640 containing [^{3}H]arachidonic acid (1 mCi/L). After approximately 24 h further incubation, the cells were washed to remove extracellular [^{3}H]arachidonic acid. In panel (A), cells received either BHA (●, ○) or BHT (▲, △) at the indicated concentrations in the absence (●, ▲) or presence of 1 μg/L rTNF-α (○, △) in a total volume of 2 mL FCS-M. The medium was removed after 4 h, centrifuged and aliquots taken for determination of radioactivity. The results are expressed as percent of the mean cpm/well in control wells (13264 cpm). The data are pooled from two separate experiments and given as mean ± SD of quadruplicates. B: Time course of rTNF-α-induced release of [^{3}H]arachidonic acid. The cells were washed and received either control medium (■), 1 μg/L of rTNF-α (□); rTNF-α plus 50 μM of BHA (○) or rTNF-α plus 50 μM of BHT (△). The medium was removed at each time point, centrifuged and aliquots taken for determination of radioactivity. The results are expressed as cpm/well, and are given as mean ± SD of duplicates from one of two similar experiments. See Figures 1 and 6 for abbreviations.

One possible mechanism is that BHA induces or activates existing defense mechanisms that protect the cell against rTNF-α cytotoxicity. TNF induces expression of mRNA for manganous superoxide dismutase (33), probably as a defense against TNF-induced radical formation. Both actinomycin D and cycloheximide increased TNF cytotoxicity (Figs. 3 and 4) in agreement with previous results (34), probably due to inhibition of protein synthesis-dependent resistance against TNF cytotoxicity. Others have shown that BHA induces the glutathione *S*-transferase enzyme (30), known to be involved in the cellular defense against free radicals. This report shows that BHA protects nearly completely against rTNF-α cytotoxicity up to 1 ng/L of rTNF-α, also in the presence of cycloheximide or actinomycin D, suggesting that BHA protection is independent of mRNA or protein synthesis. The reduced protection seen at higher rTNF-α concentrations is probably explained by the increased toxicity of rTNF-α in the presence of cycloheximide (34) or actinomycin D (Figs. 3 and 4). BHA did not reduce the effect of cycloheximide or actinomycin D on protein synthesis, suggesting that it did not inhibit rTNF-α cytotoxicity in the presence of cycloheximide through overall stimulation of protein synthesis. On the contrary, BHA alone slightly inhibited protein synthesis (Table 2). Several other observations also suggest that BHA protection is independent of protein synthesis. Firstly, preincubating WEHI cells with BHA, followed by the removal of the BHA, had no effect on rTNF-α cytotoxicity (Table 1). Secondly, adding BHA as late as 8 h after rTNF-α rescued all remaining viable cells (Fig. 1).

A recent report confirms that BHA inhibits TNF cytotoxicity in L929 cells in the presence of actinomycin D (35). However, the same study also showed that BHT partially inhibited TNF cytotoxicity at a concentration (200 μM) that we have reported killed 83% of L929 cells (24). In our hands, BHT did not inhibit rTNF-α cytotoxicity (23,24), even when we used the same experimental design as Schulze-Osthoff *et al.* (35) (data not shown). We therefore speculate that different strains of L929 cells have been used.

A second possible mechanism is that BHA inhibits rTNF-α-induced cytotoxicity through its antioxidant effect, causing trapping of free radicals. Several other reports indicate that TNF stimulates oxygen radical formation (18,21,22,35). However, BHA does probably not inhibit rTNF-α-induced cytotoxicity through a nonspecific antioxidant effect, since the antioxidants BHT (24) and α-tocopherol (18,20,24) do not inhibit rTNF-α-induced cytotoxicity, and since both BHA and BHT inhibited formation of rTNF-α-induced TBARS in non-TCA precipitable material (Fig. 6). We therefore speculate that both BHA and BHT inhibit the propagation of cell peroxidation, leading to water-soluble, non-TCA precipitable end products, while only BHA can inhibit initiation and/or the accumulation of those primary peroxides leading to cell death. This is also consistent with our observation that TNF cytotoxicity is correlated with the accumulation of protein-associated TBARS, but not with the non-TCA precipitable TBARS. This shows that only some of the rTNF-α-induced TBARS are associated with rTNF-α-induced cell death,

which would also explain the nonlinear relationship observed between protein-associated TBARS and TNF toxicity (Fig. 5). Others have already shown that different antioxidants have a different ability to inhibit TNF-induced cytotoxicity (18,20,24) supporting the idea that a generally increased formation of nonenzymatically formed radicals is probably not the key event in TNF-induced toxicity.

The differential effect of BHA and BHT on rTNF-α toxicity and protein-associated TBARS is probably not due to differences in solubility or in their antioxidant efficacy, since both inhibit the accumulation of TBARS in non-TCA precipitable material (Fig. 6), and since BHT has been shown to inhibit TBARS accumulation in other cell culture systems (36). Their similar solubility also makes it unlikely that these antioxidants accumulate at different sites inside the cell. One possible explanation of these results is that BHA, in addition to its well-known peroxide scavenging effects, specifically inhibits one or more enzyme(s) involved in both protein-associated TBARS formation and rTNF-α-induced cell death, or by some other mechanism inhibits the accumulation of specific lipid peroxides leading to cell death. Arachidonic acid increased both rTNF-α-induced protein-associated TBARS and TBARS in non-TCA precipitable material, possibly due to increased substrate for oxidation. However, preincubation with arachidonic acid increases rTNF-α cytotoxicity (23). To what extent this reflects unspecifically increased oxidative damage, or the biosynthesis of oxidized metabolites of arachidonic acid that signals rTNF-α-induced cell death, is difficult to judge because of the low specificity of the TBARS assay (37).

A third possible mechanism is that BHA interferes with one of the TNF-activated signal transduction pathways. It has been reported that following the binding of rTNF-α to its receptors, the internalization of TNF occurs within approximately 2 h in L929 cells (5). Our data suggest that BHA inhibits rTNF-α-effects by interfering with some post receptor event since BHA almost completely inhibited the toxicity of a 2-h preincubation with rTNF-α (Fig. 2). This is further supported by the observations that preincubation with BHA or BHT did not down-regulate the rTNF-α receptors (data not shown), and that BHA had no effect on TNF-induced adhesion in human endothelial cells (24). However, we cannot totally exclude the possibility that BHA affects the rTNF-α-induced turnover of its receptors as observed with the free radical scavenger desferrioxamine (38). However, this seems unlikely since BHA inhibits rTNF-α cytotoxicity completely when added 2 h after rTNF-α in WEHI and L929 cells, and protects nearly all remaining viable cells when added as late as 8 h after rTNF-α (Fig. 1B). We therefore speculate that BHA inhibits some of the late rTNF-α-induced intracellular events possibly not related to the initial TNF-induced signals.

Prostaglandins are also a part of the TNF-induced signal transduction mechanism(s). Prostaglandin E_2 is a messenger molecule in the TNF-induced regulation of CSF-1 mRNA levels in HL-60 cells (39), but the involvement of prostaglandin E_2 in TNF cytotoxicity has not been established convincingly. This report, as well as others (40), have shown that rTNF-α stimulates prostaglandin E_2 synthesis and that antioxidants have different potency in inhibiting the cyclooxygenase, BHA generally being much more potent than BHT (41,42). Since indomethacin had no effect on rTNF-α-induced cytotoxicity (23), and prostaglandin E_2 itself does not cause cell death in L929 cells (43), it seems unlikely that prostaglandin E2 directly mediates rTNF-α cytotoxicity. However, prostaglandin E_2 and other compounds may increase TNF cytotoxicity through elevation of cellular cAMP levels (43). Hayakawa *et al.* (44) showed that TNF stimulates both release of arachidonic acid and prostaglandin E_2 production in sensitive, but not in resistant, L929 cells. One critical event in TNF cytotoxicity may therefore be the TNF-induced release of arachidonic acid, a conclusion in agreement with other reports (16,17,18,32,45).

Since BHA, but generally not BHT, is an inhibitor of the lipoxygenase (46), another possible explanation is that BHA inhibits rTNF-α-induced synthesis of lipoxygenase products that could be involved in signal transduction and/or peroxide formation. Haliday *et al.* (47) reported that arachidonic acid itself and its lipoxygenase metabolite 5-hydroperoxyeicosatetraenoic acid regulate *c-fos* mRNA levels in preadipocytes, and TNF-induced lipoxygenase metabolites mediate oxidative stress in TA1 cells (48). However, we could not detect TNF-induced HETE in the supernatants from L929 cells (data not shown). Since these metabolites can accumulate intracellularly and deteriorate easily during storage, we cannot totally exclude that rTNF-α-induced lipoxygenase metabolites are produced. However, we have previously shown that nordihydroguaretic acid does not inhibit rTNF-α cytotoxicity in WEHI clone 13 cells (23), which argues against lipoxygenases being involved in rTNF-α-induced cytotoxicity in these cells.

BHA, but not BHT or indomethacin, inhibited rTNF-α-enhanced release of [^{3}H]arachidonic acid from prelabeled L929 cells (Table 4 and Fig. 7). However, none of the tested antioxidants inhibited the unstimulated release seen in the absence of rTNF-α. This indicates that BHA specifically inhibits rTNF-α-enhanced release of arachidonic acid, possibly due to the inhibition of cellular phospholipase(s). This would also reduce accumulation of lysophospholipids. BHA thereby inhibits one of the rTNF-α-induced intracellular signal mechanisms. It has been shown that phospholipase inhibitors decrease TNF-induced cytotoxicity (18,20,32). However, to our knowledge, BHA has not previously been shown to directly inhibit phospholipases. It has recently been shown that transfection of a cytosolic phospholipase A_2 into resistant L929 cells makes them susceptible to rTNF-α toxicity (49), supporting that TNF-induced activation of phospholipase A_2 is a key event in TNF toxicity. This may also explain why BHA can inhibit rTNF-α-induced cytotoxicity when protein synthesis is blocked, since many cell types constituently express phospholipase activity. The serum-induced release of arachidonic acid is not cytotoxic to L929 cells (45), and TNF stimulates both arachidonic acid release as well as growth in FS-4 fibroblasts (17), indicating that enhanced arachidonic acid release alone is not sufficient to induce cyto-

toxicity. Rather, our results suggest that the TNF-induced release of arachidonic acid must be coupled to a second mechanism when cell death is induced. We speculate that this involves the enzymatic formation of oxidized lipids since protein-associated TBARS are correlated with TNF cytotoxicity (Fig. 5).

BHA rescues remaining viable WEHI and L929 cells as late as 8 h after rTNF-α addition, while the rTNF-α-enhanced release of arachidonic acid starts 1–2 h after its addition, which is prior to cell death (Fig. 7). The most likely explanation for this apparent discrepancy is that arachidonic acid release alone is insufficient to induce cell death as discussed above. If the formed oxidized lipid metabolites exert a positive feedback on the phospholipase activity, this could explain why BHA both inhibits formation of the protein-associated TBARS, and at the same time inhibits arachidonic acid release. Another possible explanation is that rTNF-α cytotoxicity requires the ongoing release of arachidonic acid, which is then effectively inhibited by BHA.

It is also possible that BHA blocks the rTNF-α-induced increase in intracellular Ca^{2+} concentration, which starts 2–3 h after rTNF-α addition (50). Dornand *et al.* (51) have reported that BHA inhibits receptor-mediated increase in intracellular Ca^{2+}. If BHA blocks the rTNF-α-induced late increase in intracellular Ca^{2+}, this could indirectly inhibit the activity of calcium-dependent enzymes, including phospholipases.

In summary, the present report indicates that BHA, but not BHT, inhibits rTNF-α-induced cytotoxicity, rTNF-α-induced release of arachidonic acid as well as rTNF-α-induced accumulation of protein-associated TBARS. The results may suggest that activation of a phospholipase followed by the specific formation of one or more oxidized lipid metabolites mediates rTNF-α toxicity. Further studies are needed to elucidate whether fatty acid peroxides formed enzymatically from arachidonic acid are the more direct mediators of TNF-induced cytotoxicity.

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Mass Determination of the Fatty Acids Released from Tannin-Stimulated Rabbit Alveolar Macrophages

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Previous studies with macrophages that had been prelabeled with [^{14}C]arachidonic acid (20:4) have shown that condensed tannin is a potent agonist for the release of arachidonic acid. However, it has not been demonstrated that the percentage release of [^{14}C]20:4 accurately reflects the metabolic activity of the endogenous 20:4 pool. In order to measure the 20:4 mass release relative to the total cellular 20:4 pool, the free fatty acids of freshly isolated alveolar macrophages were derivatized with a fluorescent reagent, and then separated and quantified by high-performance liquid chromatography. The amounts of esterified fatty acids were measured by gas chromatography of the methyl esters. Free fatty acid levels were compared to those of the total esterified plus unesterified fatty acids to determine the actual percentage released of each fatty acid. Tannin-stimulated release of 20:4 mass reflected that previously reported for the release of [^{14}C]20:4 label but at a slower rate and at a much lower percentage indicating that [^{14}C]20:4 had been incorporated into part of a more reactive pool. The specificity of the fatty acid release induced by tannin and β-1,3-glucan, a known agonist for 20:4 release, was also examined. Both agonists promoted an increase in the levels of free 20:4 and of other fatty acids. A comparison of the absolute increases of each of the fatty acids indicated that tannin caused a preferential increase in the mass of free 20:4, whereas β-1,3-glucan evoked a selective increase in the mass of 16:0.

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Resident alveolar macrophages are the principal cells responsible for the clearance and/or detoxification of inhaled airborne occupational or environmental pollutants. Interaction of alveolar macrophages with the inhaled foreign material activates the cells and initiates a sequence of events that often results in pulmonary inflammation. Condensed tannin, a polymer of monoflavanoid subunits, is one of the major water-soluble components present in the airborne dust generated from the processing of a wide variety of woody plants (1). Inhalation of tannin or tannin-containing organic dust has been shown to evoke an acute pulmonary inflammatory response. Several of the inflammatory mediators released following tannin inhalation suggested the involvement of alveolar macrophages (2). Subsequent *in vitro* studies have shown that condensed tannin can profoundly change the functional capacity of alveolar macrophages (3–8).

Among the macrophage functions found to be modulated by condensed tannin are the release and metabolism of arachidonic acid (20:4). A recent study using alveolar macrophages prelabeled with [^{14}C]20:4 demonstrated that tannin was a potent agonist for 20:4 release. Tannin stimulated the release of up to 20% of the incorporated [^{14}C]20:4 in a time- and dose-dependent manner (7). However, rabbit alveolar macrophages are not suited to long-term culture, and hence must be loaded under nonequilibrium conditions, *i.e.*, using 2 h of labeling. With such a short loading period, it was questionable whether the labeled substrate distributed sufficiently to accurately reflect the metabolic activity of the endogenous 20:4 pool it was intended to represent.

The present study addresses this question by using a sensitive, fluorescence-based method (9) to measure the release of endogenous 20:4 mass from tannin-stimulated alveolar macrophages. As the total amount of 20:4 esterified in alveolar macrophage glycerolipids can also be measured, the percentage released from the total 20:4 pool was also determined and compared to similar data previously obtained on [^{14}C]20:4-prelabeled cells (7). In addition, because the fluorescence method permits measurement of all free fatty acids simultaneously, the specificity of the fatty acid release from tannin-stimulated alveolar macrophages was examined and compared to that evoked by β-1,3-glucan, a well-characterized agonist for 20:4 release from alveolar macrophages.

MATERIALS AND METHODS

Materials. The fatty acid standards, palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4) and heptadecanoic acid (17:0) were purchased from Nu-Chek-Prep (Elysian, MN). The fluorescent probe, 9-anthryldiazomethane (ADAM) was obtained from Molecular Probes (Eugene, OR). Medium 199 RPMI 1640, fatty acid-free bovine serum albumin (BSA) and particulate β-1,3-glucan isolated from *Saccharomyces cerevisiae* were purchased from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade solvents were obtained from EM Science (Gibbstown, NJ). A 4.5 × 250 mm, 5 μm particle size ODS reverse-phase HPLC column was purchased from Beckman Instruments (Fullerton, CA). Tissue culture plates were obtained from Costar (Cambridge, MA).

Isolation of cotton bract tannin. Condensed tannin was isolated as previously described (10) from the presenescent bracts of Acala SJ-5 cotton grown in California in 1982. The bracts were a generous gift of Dr. Robert Jacobs of Cotton, Incorporated (Raleigh, NC). The purified tannin was stored as a desiccated, lyophilized powder at –20°C. Fresh solutions were made immediately before use by dissolving the tannin in calcium- and magnesium-free Hank's balanced salt solution (HBSS), and the concentration of tannin was deter-

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Abbreviations: ADAM, 9-anthryldiazomethane; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography.

mined by the spectrophotometric method of Bell and Stipanovic (11).

Preparation of particulate β-1,3-glucan. Particulate β-1,3-glucan was prepared by suspension in HBSS at 5 mg/mL and was subjected to tip sonication just prior to use to separate any adherent particles. Following sonication, the number of particles/mL was measured on a hemacytometer.

Isolation of rabbit resident alveolar macrophages. Resident alveolar macrophages were obtained by bronchoalveolar lavage of healthy, adult New Zealand white rabbits as previously described (12). Cell differentials performed on Wright's-stained cytospin preparations revealed that the percentage of macrophages exceeded 95% in all cases. The cells were resuspended at a concentration of 1.5×10^6 cells/mL in culture medium (a 1:1 mixture of Medium 199 and RPMI 164 supplemented with 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). One-mL aliquots of the macrophage suspension were added to each well of 6-well tissue culture plates, and the plates were incubated for 1 h at 37°C in a fully-humidified atmosphere of 95% air/5% CO_2 to permit the macrophages to adhere. The wells were then washed three times to remove any nonadherent cells, then 1 mL of mixed media +0.1% BSA was restored to each well.

Quantification of total phospholipids and esterified fatty acids in macrophages. Lipids were extracted from 1×10^7 freshly isolated rabbit alveolar macrophages by the method of Bligh and Dyer (13). After removal of the solvent under nitrogen, the lipids were redissolved in 1 mL of chloroform/methanol (2:1, by vol). Aliquots of this extract were assayed for lipid phosphorus by the method of Bartlett (14) and for total esterified fatty acids by transesterification with 0.2 N methanolic NaOH and gas chromatography analysis of the methyl esters in the presence of an internal standard.

Determination of the time and dose dependence for tannin-mediated 20:4 release. To define the time dependence of tannin-mediated 20:4 release, adherent macrophages were incubated at 37°C in a fully-humidified atmosphere of 95% air/5% CO_2 in 1 mL of culture medium containing 100 µg/mL tannin. After 30, 60, 90 and 120 min of incubation, the cells were freed from the plates by scraping with a silicone policeman. The suspended cells from 2–4 wells were combined, transferred to acid-washed 50-mL glass tubes and 2.000 µg of 17:0 was added to each tube to serve as an internal standard. The fatty acids were then extracted by the method of Bligh and Dyer (13). Cells extracted immediately after addition of the tannin-containing medium defined the basal level of 20:4 at the onset of the study. Following extraction, the organic solvent was removed under nitrogen and the fatty acids redissolved in 200 µL of methanol and stored at -70°C until derivatization and analysis.

To define the dose dependence of tannin-mediated 20:4 release, adherent macrophages were incubated at 37°C in a fully-humidified atmosphere of 95% air/5% CO_2 in 1 mL of culture medium containing 25, 50, 100 or 200 µg/mL tannin. After 90 min, the fatty acids were extracted and stored until analysis, as described above. Two sets of controls were run in parallel accompanying each experiment. Macrophages incubated with culture medium alone served as controls to define the basal release of 20:4. Wells containing culture medium, but no cells, served to define the level of fatty acids present in the medium.

Comparison of fatty acid release by tannin and β-1,3-glucan. To compare the release of all fatty acids from macrophages stimulated by tannin with that stimulated by β-1,3-glucan, adherent macrophages were stimulated with either culture medium containing 100 µg/mL tannin or 2×10^7 particles/mL of β-1,3-glucan for 90 min at 37°C in a fully-humidified atmosphere of 95% air/5% CO_2. The fatty acids were then extracted and stored until analysis as described above.

Separation and quantification of the free fatty acids. To prepare fluorescent derivatives of the extracted free fatty acids, a 200-µL aliquot of 10 mg/mL ADAM in methanol was added to the extracted fatty acids in methanol prepared as described above. The reaction tubes were purged with nitrogen, sealed and incubated for four hours in the dark at room temperature. Samples were then stored at -20°C until analysis. All samples were analyzed within 24 h after completion of the derivatization. Preliminary studies demonstrated that the fluorescent fatty acid derivatives were stable for at least this period of time.

Separation and quantification of the fluorescently-derivatized fatty acids was performed by HPLC using the method described by Nakagawa and Waku (9). Aliquots of 100 µL of the fluorescently-derivatized fatty acids were subjected to chromatography on a Waters Maxima HPLC system (Milford, MA) equipped with an in-line model 470 fluorescence detector. Separation of the derivatized fatty acids was achieved by chromatography on an ODS reverse-phase column with isocratic elution using a mobile phase of acetonitrile/isopropanol/water (90:9:1, by vol) at a flow rate of 1.6 mL/min. The derivatized fatty acids were detected by monitoring fluorescence emission at 412 nm resulting from excitation at 365 nm. The elution profiles of a standard mixture of ADAM-derivatized fatty acids were used to identify the individual fatty acid derivatives present in the cell extracts. Mass values for each of the fatty acids were determined by comparison of the integrated peak area of each fatty acid derivative and that of the derivatized internal standard, 17:0.

Statistical analysis. Comparisons of statistical significance were made using the paired Student's *t*-test. A $P < 0.05$ was taken as the limit to indicate statistical significance.

RESULTS

Total phospholipid and esterified fatty acids in alveolar macrophages. Total lipid phosphorus was determined to be 64.0 ± 2.3 nmol/10^6 cells (n = 4), a value very similar to that reported by Sugiura *et al.* (15). Fatty acid methyl esters obtained by methanolysis of the total lipid extract, amounted to 106.6 ± 3.2 nmol/10^6 cells (n = 4). About 98% of these consisted of the five predominant

fatty acids 16:0, 18:0, 18:1, 18:2 and 20:4. While the most abundant esterified fatty acid was 18:1, which comprised 33.0% of the total, the rest were present in nearly equal amounts of 15 to 17% each (Table 1).

Free fatty acid in alveolar macrophages. The free fatty acids in freshly isolated or tannin-stimulated rabbit alveolar macrophages were extracted, derivatized with a fluorescent probe, separated by HPLC (as illustrated in Fig. 1). In unstimulated cells, the mass of free fatty acids present ranged from a low of 12 ± 4 ng/10^6 cells for 20:4 to a high of 1200 ± 80 ng/10^6 cells for 16:0 (Table 1). When expressed as a percentage of the total amount of free fatty acids present in the macrophages, 16:0 accounted for approximately 56% of the total free fatty acids; 18:2, 18:1 and 18:0 represented 11, 17 and 16% of the total, respectively; and 20:4 accounted for only 0.5%. These values agree well with those previously reported for freshly isolated rabbit alveolar macrophages by Nakagawa and Waku (9).

When the amount of unesterified fatty acid was expressed as nmol/10^6 cells and compared to the total nmol/10^6 cells present in the alveolar macrophages (Table 1), it was found that approximately 7% of the total fatty acids present in the macrophages were unesterified. Individually, the relative amount of each free fatty acid present in the free fatty acid pool varied from 20:4, of which only 0.2% was unesterified, to 16:0, of which nearly 23% was present as free fatty acid. The relative amounts of the other free fatty acids (18:2, 18:1 and 18:0) ranged from 4–7% of their total amounts.

TABLE 1

Free Fatty Acid Composition of Freshly Isolated Alveolar Macrophages

Fatty acids	FFA[a] ng/10^6 cells	FFA[a] nmol/10^6 cells	Esterified FA[b] nmol/10^6 cells
16:0 (% free)[c]	1200 ± 80	4.69 ± 0.30 (22.7 ± 1.4%)	16.0 ± 0.8
18:0 (% free)	409 ± 63	1.44 ± 0.22 (7.5 ± 1.1%)	17.8 ± 0.4
18:1 (% free)	440 ± 41	1.56 ± 0.14 (4.2 ± 0.4%)	35.2 ± 0.7
18:2 (% free)	274 ± 57	0.98 ± 0.20 (5.2 ± 1.1%)	17.8 ± 0.6
20:4 (% free)	12 ± 4	0.04 ± 0.01 (0.2 ± 0.0%)	17.9 ± 1.1
Total[d] (% free)	2340 ± 160	8.69 ± 0.59 (6.6 ± 0.4%)	106.6 ± 3.2

[a]The values shown are the means ± SEM mass for each of the individual fatty acids (FA) determined on six independent preparations of alveolar macrophages. FFA, free fatty acids.

[b]The values shown are the means ± SEM nmol for each of the individual fatty acids determined on four independent preparations of alveolar macrophages.

[c]The percentage of each fatty acid present in the unesterified form calculated as (nmol FFA/10^6 cells) ÷ (total FFA + esterified FA).

[d]The other fatty acids, *e.g.*, 16:1, 20:5, etc., were present in low abundance (>2% of the total combined) and are not reported. Their contribution to the total fatty acids of the macrophage are, however, reflected in the total.

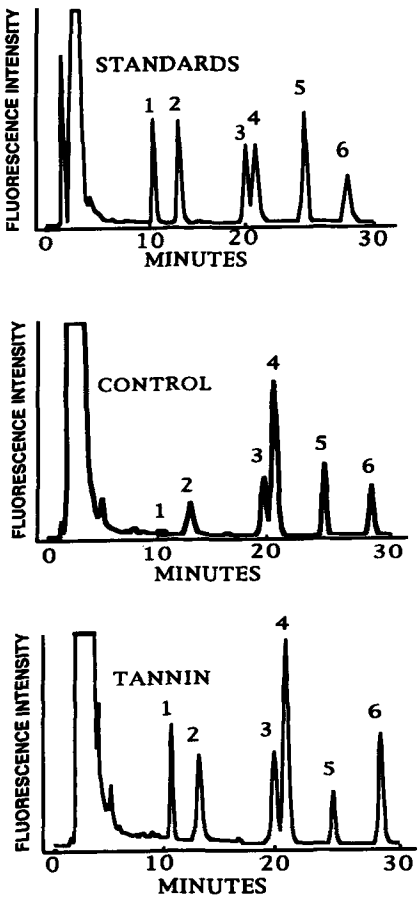

FIG. 1. Separation of fatty acid 9-anthryldiazomethane esters by reverse-phase high-performance liquid chromatography. Shown in the top panel is a chromatogram of a mixture of standard fatty acids following derivatization and chromatography on a reverse-phase ODS column. The mixture has resolved into individual fractions corresponding to 20:4 (1); 18:2 (2); 18:1 (3); 16:0 (4); 17:0 (5); and 18:0 (6). Shown in the center and bottom panels are the profiles of the fatty acids extracted from 4.5 × 10^6 control or tannin-stimulated (100 μg/mL) alveolar macrophages, respectively. In each case, 17:0 was added to each sample prior to extraction to serve as an internal standard to calculate recovery and quantitate the mass of each fatty acid.

Effect of alveolar macrophage adherence on free fatty acid levels. Since all subsequent experiments utilized macrophages that had been allowed to adhere to tissue culture plates, it was necessary to determine whether any changes occurred as a result of adherence. Freshly isolated macrophages contained 8.69 ± 0.59 nmol free fatty acids/10^6 cells compared to 8.43 ± 0.62 nmol free fatty acids/10^6 cells found in macrophages after one hour of adherence, a difference that was not statistically significant ($P = 0.74$). Adherence did, however, alter the mass distribution among the five free fatty acids detected in rabbit alveolar macrophages, as shown in Figure 2. Comparison of the mass of individual free fatty acids in freshly isolated cells with that in cells after one hour of adherence revealed that the mass of all three un-

saturated fatty acids increased. The mass of free 20:4 increased from 0.04 ± 0.1 to 0.13 ±0.03 nmol/10^6 cells, while free 18:2 increased from 0.98 ± 0.20 to 1.67 ± 0.17 nmol/10^6 cells, and free 18:1 increased from 1.56 ± 0.14 to 1.79 ± 0.26 nmol/10^6 cells. However, the increase in these free fatty acid levels reached statistical significance only for 18:2 ($P = 0.023$). In contrast, the masses of both saturated fatty acids, 18:0 and 16:0, decreased from 1.44 ± 0.22 to 1.11 ± 0.15 nmol/10^6 cells and 4.69 ± 0.30 to 3.75 ± 0.26 nmol/10^6 cells, respectively, but only the decrease in 16:0 was statistically significant ($P = 0.05$).

Time dependence of tannin-mediated release of arachidonic acid. Exposure of alveolar macrophages to tannin resulted in the time-dependent release of 20:4. Macrophages challenged with 100 µg/mL tannin for 2 h released 1.26 ± 0.23 nmol 20:4/10^6 cells as compared to the basal release of 0.08 ± 0.04 nmol 20:4/10^6 cells observed over a 2-h period from adherent, unstimulated control macrophages. As illustrated in Figure 3, 20:4 release was initially modest, with only 15% of the maximal release observed 30 min after exposure to tannin. This was followed by a rapid release over the next 30 min so that by 1-h post-exposure, 95% of the maximal release of 20:4 had occurred. This lag in the time course for endogenous 20:4 release differed from the previously reported determination of the time dependence of 20:4 release based on the measurement of the liberation of [^{14}C]20:4 from prelabeled alveolar macrophages (7). The time dependence for the release of [^{14}C]20:4 is included in Figure 3 to allow comparison with the time dependence of endogenous 20:4 release. As illustrated, the release of [^{14}C]20:4 was significantly greater and faster than that observed for the release of 20:4 mass determined in the present study. While only 15% of the maximal release of endogenous 20:4 mass occurred within 30 min after exposure, 65% of the maximal [^{14}C]20:4 release occurred within that time. However, measurements made by either method demonstrated that arachidonate release reached a plateau after 60 min of tannin stimulation.

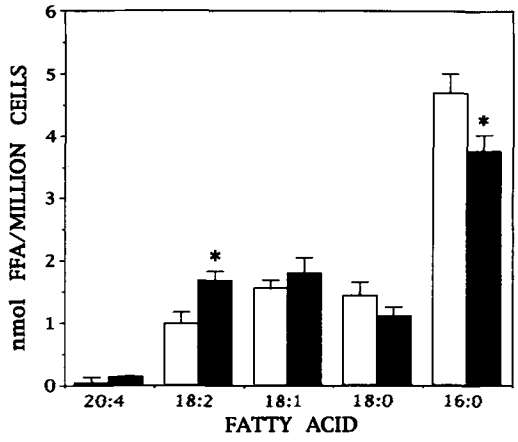

FIG. 2. Comparison of the mass of free fatty acid (FFA) in freshly-isolated, nonadherent alveolar macrophages and macrophages one hour after adherence. The levels of free fatty acids, expressed as nmol of free fatty acid/10^6 cells are shown for both freshly-isolated, nonadherent alveolar macrophages (open bars) and macrophages 1 h after adherence (closed bars). The values shown are the means ± SEM for five independent preparations. Levels of free fatty acid in adherent macrophages that differed statistically ($P < 0.05$) from those in freshly-isolated nonadherent macrophages are indicated by an asterisk.

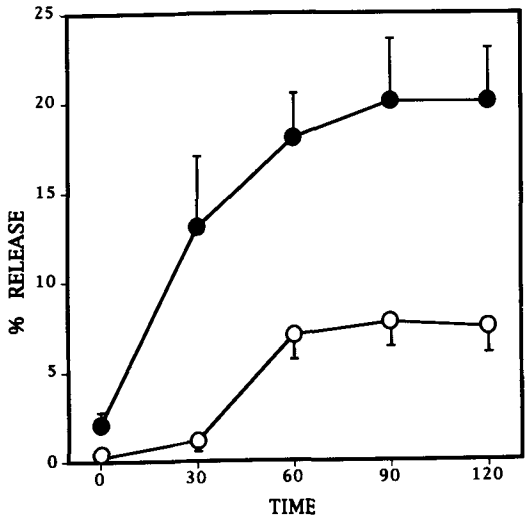

FIG. 3. Time course for tannin-mediated release of 20:4. Shown is the time-dependent increase in the mass of free 20:4 in alveolar macrophages stimulated with 100 µg/mL tannin (open circles). Each value is the mean ± SEM from four independent preparations. For comparison, the previously published (7) time-dependent release of [^{14}C]20:4 from alveolar macrophages prelabeled for 2 h with [^{14}C]20:4 is also shown (closed circles). Although the actual percentage release was significantly greater ($P < 0.05$) at all points for the radioactive 20:4 release, a comparison of 20:4 release expressed as a percentage of the maximal release, *i.e.*, the release determined at 90 min post-exposure to tannin measured by these two methods, was different only at 30 min ($P < 0.001$).

Dose dependence of tannin-mediated release or arachidonic acid. Previous studies had indicated an unusual dose dependence for the tannin-mediated release of [^{14}C]20:4 from prelabeled rabbit alveolar macrophages (7). No release of [^{14}C]20:4 above basal levels was observed at tannin concentrations below 25 µg/mL. Although the release of [^{14}C]20:4 was consistently higher, the pattern was similar to the dose-dependent release of endogenous 20:4 measured 90 min after exposure to tannin. No increase in the mass of free 20:4 occurred with tannin concentrations at or below 25 µg/mL, but near-maximal release occurred at 100 µg/mL (Fig. 4). The similarity between these two different methods of assessing the dose dependence of tannin-stimulated arachidonate release is also apparent by comparison of the EC_{50} for each method. The EC_{50} for tannin-mediated 20:4 release based on mass was 65 µg/mL as compared to the previously reported EC_{50} of 75 µg/mL based on the release of incorporated [^{14}C]20:4.

Comparison of the specificity of fatty acid release evoked by tannin and β-1,3-glucan. Because the fluorescence technique permitted the quantification of all free fatty acids released, it was possible to assess the specificity of the release of fatty acids in response to tannin

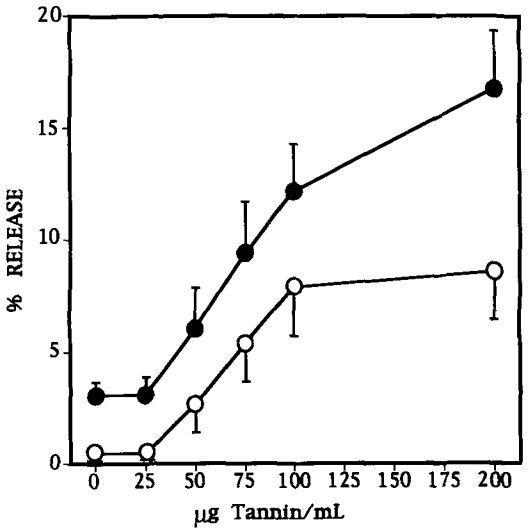

FIG. 4. Dose dependence of the tannin-mediated release of 20:4. Shown is the dose-dependent increase in the mass of free 20:4 in alveolar macrophages 90 min after the onset of exposure to the indicated concentrations of tannin (open circles). Each value is the mean ± SEM from five independent alveolar macrophage preparations. For comparison, the previously published (7) dose-dependent release of [^{14}C]20:4 from alveolar macrophages prelabeled for 2 h with [^{14}C]20:4 is also shown (closed circles). The percentage release of [^{14}C]20:4 was significantly greater ($P < 0.05$) than the release of endogenous 20:4 for all tannin concentrations.

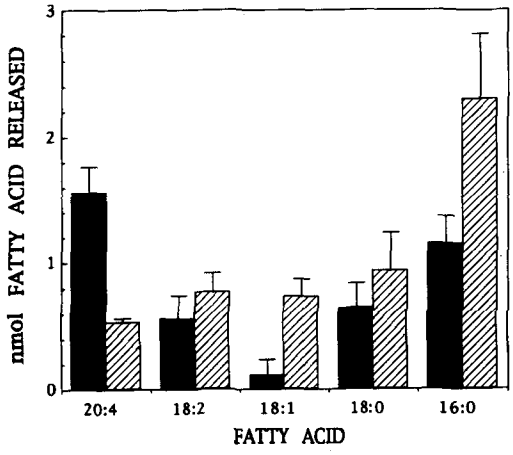

FIG. 5. Comparison of the absolute increases in the mass of free fatty acids in alveolar macrophages stimulated with tannin or β-1,3-glucan. The absolute increase in the mass of each free fatty acid, expressed as nmol of free fatty acid/10^6 cells, is shown for alveolar macrophages stimulated for 90 min with 100 μg/mL tannin (black bars) or 2 × 107 particles/mL of β-1,3-glucan (hatched bars). The levels were calculated by subtraction of the mass of each free fatty acid in control macrophages from the mass of the corresponding fatty acid in the stimulated cells. The values shown are the means ± SEM for eight independent preparations. The increase in free 20:4 was greater ($P < 0.05$) than that of 18:2, 18:1 and 18:0 in tannin-stimulated cells. The release of 16:0 was greater ($P < 0.05$) than that of all the other fatty acids released by β-1,3-glucan.

TABLE 2

Mass of Free Fatty Acids in Control, Adherent Rabbit Alveolar Macrophages and Adherent Macrophages Challenged with Tannin or β-1,3-Glucan[a]

Fatty acid	Control	Tannin	β-1,3-Glucan
16:0	3.44 ± 0.35	4.58 ± 0.40[b] (1.14 ± 0.23)[c]	5.72 ± 0.57[b] (2.29 ± 0.52)
18:0	0.92 ± 0.11	1.57 ± 0.21[b] (0.64 ± 0.19)	1.85 ± 0.31[b] (0.93 ± 0.31)
18:1	1.82 ± 0.18	1.93 ± 0.26 (0.11 ± 0.12)	2.55 ± 0.28[b] (0.73 ± 0.14)
18:2	1.70 ± 0.26	2.25 ± 0.28[b] (0.55 ± 0.19)	2.47 ± 0.28[b] (0.77 ± 0.15)
20:4	0.18 ± 0.07	1.72 ± 0.24[b] (1.55 ± 0.22)	0.71 ± 0.10[b] (0.53 ± 0.04)
Total	8.06 ± 0.90	12.06 ± 1.09[b] (4.00 ± 0.65)	13.29 ± 1.24[b] (5.23 ± 0.99)

[a]The values shown are the means ± SEM mass (nmol/10^6 cells) for each of the individual fatty acids in control macrophages after two hours of incubation in culture medium (Control), culture medium plus 100 μg/mL tannin (Tannin) or culture medium plus 20 × 10^7 particles of β-1,3 glucan. The values represent the average of eight independent preparations of alveolar macrophages.

[b]Values were statistically significantly greater ($P < 0.05$) than the corresponding value in the control cells.

[c]The mean ± SEM nmol/10^6 cells increase for each fatty acid over the level detected in control macrophages.

or any other agonist. Challenge of alveolar macrophages with 100 μg/mL tannin resulted in a 50% increase in the level of total free fatty acids. As seen in Table 2, tannin-stimulated macrophages released more 20:4 than any other fatty acid, 1.55 nmol/10^6 cells, which was significantly greater ($P > 0.05$) than the release of other free fatty acids except 16:0. For comparison, the fatty acid release induced by β-1,3-glucan was also determined. β-1,3-Glucan is a well-characterized component of zymosan, a yeast cell fragment responsible for liberation and secretion of arachidonate from alveolar macrophages (16). As also shown in Table 2, macrophages stimulated with 2 × 10^7 β-1,3-glucan particles/mL responded with a 65% increase in free fatty acids, similar in total mass to that resulting from tannin stimulation. However, the profile of free fatty acids was quite different (Fig. 5). Whereas tannin caused a release of similar amounts of saturated and unsaturated fatty acids, β-1,3-glucan stimulated a release of saturated fatty acids, which was much greater than the release of unsaturated fatty acids. The most predominant fatty acid released by β-1,3-glucan was 16:0, which at 2.29 nmol/10^6 cells, was significantly ($P < 0.05$) more than any other fatty acid.

Comparisons based on the percentage increase in the level of any free fatty acid are dependent upon the basal level of each fatty acid. Although tannin caused a 10-fold, and β-1,3-glucan caused a 4-fold, increase in free 20:4, referring to their release in this way can be specious because the basal levels of free 20:4 are so low relative to the other fatty acids. To achieve a more meaningful indication of the selectivity of fatty acid release in

response to agonist stimulation, we have chosen in Figure 5 to compare the increase in actual mass of each fatty acid over the basal levels in the agonist-stimulated cells. Depicted in this way, it is apparent that the increase in mass of 20:4 was less than that for all the other fatty acids in β-1,3-glucan-stimulated macrophages. Thus, despite the fact that β-1,3-glucan induced a fourfold increase in the level of free 20:4, it did not induce a selective release of 20:4. In fact, the level of free 16:0, which increased 70% relative to controls, represented the greatest mass release of any fatty acid. A quite different picture emerged from the analysis of the mass changes of the fatty acids in tannin-treated macrophages. In these cells, the largest increase in mass was observed for 20:4, which was significantly greater than that observed for 18:2, 18:1 or 18:0. These data indicate that, unlike β-1,3-glucan, tannin evoked a fatty acid release which was preferential for 20:4.

DISCUSSION

The mechanisms and factors involved in the agonist-dependent release of 20:4 levels have been studied in many cell types by examining the levels of radioactive 20:4 released from prelabeled cells. In such studies, it is important to know when the added radioactive arachidonate achieved isotopic equilibrium within the phospholipid pools of the cells, and if not, what effect this isotropic heterogeneity would have on stimulated release. Several factors can contribute to heterogeneous 20:4 distribution within cellular lipid pools. First, it is well established that the rates at which different phospholipid classes are labeled with exogenous radioactive 20:4 are quite different. Phosphatidylcholine and phosphatidylinositol are labeled much more rapidly than phosphatidylethanolamine (17). Second, within the phosphatidylcholine and phosphatidylethanolamine pools, the diacyl phospholipids are labeled more rapidly than either the alkylacyl or alkenylacyl phospholipids (18). Finally, the rates at which the same phospholipid present in the membranes of different subcellular organelles are labeled may also differ (19). Thus, it was not unexpected to find that the level of [^{14}C]20:4 released from the radiolabeled pools was different than the endogenous 20:4 release.

Several conclusions can be drawn from the measurements of endogenous fatty acid mass released from alveolar macrophages, especially in relation to the amounts of each in free and esterified pools. Upon consideration of the fatty acid levels in basal nonadherent cells, the low level of free 20:4 and high level of free 16:0 are most striking. Although 20:4 and 16:0 comprise similar amounts of the total esterified fatty acids found in the macrophage, 16.8 and 15%, respectively, their levels as free fatty acids are quite differently controlled in the cell (20). Although the low level of free 20:4 in alveolar macrophages was anticipated from earlier work, the finding that 23% of the cells' 16:0 was in the unesterified form was unforeseen. One important factor to be considered in this regard is that the pulmonary surfactant fluid in which the alveolar macrophages reside is markedly enriched in dipalmitoyl phosphatidylcholine. The high levels of free 16:0 in the alveolar macrophages might be the result of dipalmitoyl phosphatidylcholine ingestion and degradation (21,22). Furthermore, free 16:0 represents more than 50% of the free fatty acids in this lipid-rich milieu (data not shown). Thus, until their removal from the lavage fluid, the alveolar macrophages are exposed to substantial amounts of 16:0.

The decrease in the levels of free saturated fatty acids and increase in free unsaturated fatty acids observed during the process of cell adherence is consistent with the conclusion that cell adherence leads to a increased turnover of the phospholipid fatty acids with a release of unsaturated fatty acids such as 20:4. This supports the previous observation by Kousan and colleagues (23) that adherence of alveolar macrophages to a plastic surface results in the release of 20:4. A more recent study of monocyte adherence by Lefkowith and colleagues (24) suggested that adherence and spreading not only results in the activation of cellular phospholipases but also the release of 20:4 is a necessary and integral part of the adhesion process. Inhibition of the phospholipases resulted in decreased adherence and spreading.

The difference in rate for the release of [^{14}C]20:4 and endogenous 20:4 mass suggest that at least two pools of 20:4 exist within the alveolar macrophage that differ in the rate at which 20:4 is released following cell stimulation. The [^{14}C]20:4 incorporated into the alveolar macrophages during a two-hour labeling period appears to enter a more "reactive" pool since the rate at which the [^{14}C]20:4 is released is faster and greater than the percent of endogenous 20:4 mass released from the cells' lipid pools. Several previously published reports (25–27) have suggested the existence of different intracellular pools of 20:4 which are distinguishable on the basis of the rates at which they either incorporate or release 20:4. Fonteh and Chilton (28) have recently reported that the increase in extracellular 20:4 released from antigen-activated mast cells was more rapid than the increase in free intracellular 20:4 or 20:4 metabolites. The extracellular 20:4 also differed in specific radioactivity from free intracellular 20:4 or 20:4 metabolites. They concluded that the extracellular 20:4 and intracellular free 20:4 arose from mobilization of different pools of 20:4. If a similar situation also occurs in rabbit alveolar macrophages, it could explain the discrepancy between the initial rates of 20:4 release from the tannin-stimulated macrophages measured by release of pre-incorporated [^{14}C]20:4 and the release of total 20:4 mass measured. The previously reported measurement of [^{14}C]20:4 release from prelabeled macrophages quantified only the extracellular [^{14}C]20:4 while the mass determinations used in the present study measured the combined total mass of free intracellular and extracellular 20:4. However, it is interesting to note that tannin-mediated arachidonate release from [^{14}C]20:4 prelabeled cells was similar to that from endogenous 20:4 in terms of dose response and time dependence.

β-1,3-Glucan is presumed to stimulate the deacylation of phospholipids by activation of phospholipase A_2 (29) and is known to have little or no effect on the activity of

the enzymes involved in reacylation (7). However, as is apparent from the release of saturated fatty acids at a greater rate than the unsaturated fatty acids, it appears that the glucan-stimulated deacylation activity is not selective for fatty acids located in the *sn*-2 position. On the contrary, comparison of the amounts of individual fatty acids released in response to β-1,3-glucan reveals that 16:0 was selectively released. This selectivity of β-1,3-glucan-stimulated fatty acid release was not observed in cells prelabeled with [^{14}C]20:4 and [^{14}C]16:0 (16) and requires further study.

While β-1,3-glucan stimulated a significant release of all fatty acids, tannin-mediated release was significant for all fatty acids, except 18:1, which remained at a virtually constant level in macrophages challenged with tannin. As 18:1 is twice as abundant as any other macrophage fatty acid at ~37% of the total, it is remarkable that it is the least affected by tannin (Table 2).

Finally, comparison of tannin- and β-1,3-glucan-stimulated macrophage free fatty acid levels is consistent with the hypothesis that the mechanism responsible for fatty acid release is different for these two agonists. Whereas β-1,3-glucan appears to stimulate phospholipase A activity, tannin may increase fatty acid levels by inhibiting reacylation (7). The fact that tannin induces the greatest increase in the mass of free 20:4 is consistent with our previous observation that the inhibition of reacylation of fatty acids in tannin-treated macrophages was most pronounced for 20:4 (30). Together, these results suggest that rabbit alveolar macrophages may contain a 20:4-specific acyl-CoA synthetase as has been reported for human platelets (31) and that inhibition of this enzyme by tannin is responsible for the selective increase in free levels of 20:4. Further studies are currently underway to investigate this possibility.

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Essential Fatty Acids Alter the Activity of Manganese-Superoxide Dismutase in Rat Heart

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The effects of oil-derived dietary essential fatty acids on the activities of mitchondrial Mn-SOD (manganese-superoxide dismutase) and cytosolic cupric zinc-superoxide dismutase (Cu/Zn-SOD) were investigated in rat heart. A control group of rats was fed a stock diet for 29 d, and a second group was fed on a fat-free diet. Three other groups were fed fat-free diets that were supplemented with (i) borage oil, which is rich in linoleic (18:2n-6) and γ-linolenic (18:3n-6) acids, (ii) fungal oil, which is rich in γ-linolenic, but low in linoleic acid, or (iii) evening primrose oil, which is rich in linoleic acid and low in γ-linolenic acid. An increase in the percentage composition of arachidonic acid (20:4n-6) in both the choline and ethanolamine phospholipids, together with a decrease in linoleic acid in ethanolamine phospholipids, were found in heart membranes after feeding the rats with diets containing borage oil or fungal oil as compared to those fed the stock diet. The respective activities of Mn-SOD in rats fed the borage or fungal oil diets were also significantly higher than in rats fed the stock diet alone. No change in cytosolic Cn/Zn-SOD activity was observed. Dietary supply of linoleic acid-rich evening primrose oil resulted in an increased proportion of choline phospholipid linoleic acid without any changes in arachidonic acid content or in the activity of Mn-SOD. By contrast, a reduction in the activity of Mn-SOD was detected in rats fed a fat-free diet. These results show that the activity of heart mitochondrial Mn-SOD is influenced by dietary essential fatty acids, whereas the activity of cytosolic Cu/Zn-SOD remained unaffected.
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Superoxide radicals are physiologically generated in mammalian cells during the transport of electrons along the mitochondrial respiratory chain (1,2) through metabolic processes catalyzed by oxidative metallo-enzymes and through nonenzymatic oxidoreductive reactions (3,4). The radicals are dismutated to hydrogen peroxide and molecular oxygen by mitochondrial matrix-soluble manganese-superoxide dismutase (Mn-SOD) and cytosolic soluble cupric zinc-superoxide dismutase (Cu/Zn-SOD) (5). The enzymatic dismutation of these radicals at their sites of formation offers protection to cellular structures from peroxidative damage (4).

During myocardial ischemia, the activity of mitochondrial Mn-SOD is reduced by approximately 50%, and reperfusion does not restore the activity of the enzyme (6). A quantitatively similar reduction in Mn-SOD activity has also been reported in the iris ciliary body during endotoxin-induced acute ocular inflammation, where activity is restored within seven days as the inflammatory reaction declines (7). It is not known how and why the activity of Mn-SOD is reduced, although it is known that under both myocardial ischemia and ocular inflammation, mitochondria show structural alterations, impairment of respiratory function, inhibition of SH-dependent enzymes and changes in calcium transport kinetics (7–9). In addition, membrane-bound arachidonate is released and peroxidized to eicosanoids (10–12). Release of catecholamines from membrane vesicles (13,14), activation of xanthine dehydrogenase to oxidase (3,15) and generation of superoxide radicals (16–18) have also been detected in the heart during ischemia. Membrane lipid peroxidation, inhibition of ATPase, release of catecholamines and impairment of respiratory mitochondrial function have been observed during cardiotoxicity induced by adriamycin (19,20), a drug known to generate superoxide radicals in the mitochondria, following complexation with iron (21,22). Furthermore, iron-induced lipid peroxidation of isolated myocardial mitochondria is inhibited by addition of superoxide dismutase (SOD) and catalase (23).

Most of the above data point toward peroxidative damage and suggest that under oxidative stress the antioxidant defenses of the heart are unable to dispose of oxygen radicals and thus cannot protect against oxygen-induced toxicity. The activities of the protective antioxidant enzymes are comparatively low in the heart (6,24,25). Since cardiac phospholipid composition can be altered by dietary fatty acids with associated changes in the activities of some enzymes (26–28), the present study was undertaken to investigate the effects of diets containing borage oil (BOO), fungal oil (FGO) and evening primrose oil (EPO) as sources of essential fatty acids on the activities of mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD in rat heart tissue.

MATERIALS AND METHODS

Reagents. Xanthine, buttermilk xanthine oxidase, *p*-iodonitrotetrazolium violet (INT), SOD and bovine serum albumin were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Inorganic salts were of the AnalaR Grade and were obtained from British Drug House (Poole, Dorset, United Kingdom).

Animals and diets. Fifteen six-week-old female Wistar rats were obtained from Bantin & Kingman Ltd. (Hull,

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Abbreviations: BOO, borage oil; CPL, choline phospholipids; Cu/Zn-SOD, cupric zinc-superoxide dismutase; EPO, evening primrose oil; EPL, ethanolamine phospholipids; FFD, fat-free diet; FGO, fungal oil; HPLC, high-performance liquid chromatography; INT, *p-iodonitrotetrazolium violet*; Mn-SOD, manganese-superoxide dismutase.

TABLE 1

Composition of Diets[a]

Nutrient	Fat-free diet (wt%)	Stock diet[b]
Protein	15.3	14.7
Fat	0.0	2.6
Fiber	4.6	5.3
Carbohydrate	70.5	61.5
Ash	4.5	5.9
Moisture	5.0	10.0

[a]Vitamin and mineral composition: vitamin A, 2.9 mg/kg; vitamin E, 97 mg/kg; vitamin C, 47.6 mg/kg; iron 65.1, mg/kg; copper, 11.6 mg/kg; zinc, 27.2 mg/kg; selenium, 69.9 mg/kg; manganese 50 mg/kg.
[b]Percentage of main fatty acids in stock diet: palmitic acid, 0.32%; oleic acid, 0.76%; linoleic acid, 0.71%; α-linolenic acid, 0.06%; arachidonic acid, 0.13%.

United Kingdom) and fed one of five diets for 29 d. Diet 1 was a stock rodent diet RM1 (see Table 1) supplied by Special Diet Services Ltd. (Witham, Essex, United Kingdom). Diet 2 was a fat-free rodent diet (FFD), also supplied by Special Diet Services Ltd. (see Table 1). Diets 3, 4 and 5 consisted, respectively, of FFD 2 and EPO *(Oenothera biennis)* to provide a linoleic acid-enriched Diet 3, BOO *(Borago officinalis)* to provide a linoleic acid and γ-linolenic acid-enriched Diet 4, and FGO *(Mucor javanicus)* to provide a low linoleic acid and high γ-linolenic acid-enriched Diet 5. Each oil was added at 5% of the total dietary energy. The fatty acid compositions of the oils before diet mixing are shown in Table 2. The synthetic antioxidant 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene) was added, at 50 mg/100 g, to the oils. Animals were housed three per experimental group in grid bottomed cages at a mean room temperature of 21 ± 1.5°C and a light/dark cycle of 14 h light and 10 h dark.

Sacrifice of animals and collection of tissues. Animals were euthanized with diethyl ether after 29 d on the diets. The heart was immediately removed and placed on ice. About 100 mg of tissue was excised from the apex of the heart and homogenized in 4 mL of 0.25 M sucrose in 0.1 M potassium phosphate buffer (pH 7.2) at 4°C. Homogenization was done using a borosilicate tube and a Teflon plunger with a clearance of 0.20 mm at 2,000 rpm. The remaining heart was frozen at −20°C for subsequent fatty acid analysis.

Subcellular fractionation. Exactly 1.0 mL of the homogenate was removed and kept at −20°C for enzyme assays. The remaining 3 mL were centrifuged at 37,000 × g at 4°C for 20 min using a Beckman model J2-21 centrifuge (Beckman, Palo Alto, CA). At the end of the run, the mitochondria-rich pellet was separated from the cytosolic supernatant, washed twice and suspended in 2 mL of sucrose phosphate buffered medium (pH 7.2). Succinate dehydrogenase was used as a mitochondrial marker enzyme to test the effectiveness of the subcellular fractionation of the homogenate into a mitochondria-rich fraction and a cytosolic supernatant.

Protein determination. The concentration of protein in 100-μL aliquots of heart homogenates, and of mitochondrial and cytosolic fractions, were estimated by the biuret method (29).

Tests for SOD. In preliminary experiments, pretreatment of heart mitochondrial suspension (200 μL) with 150 μL of chloroform/ethanol (2:1, vol/vol) abolished the activity of superoxide dismutase. In separate tests, addition of 3 mM potassium cyanide to the assay mixture did not exert a significant inhibitory effect on the activity of mitochondrial SOD. In contrast, the activity of the cytosolic SOD was inhibited by 3 mM potassium cyanide, but remained unaffected by the chloroform/ethanol mixture. This indicated that the mitochondrial fraction contained mainly the chloroform/ethanol inactivated, cyanide insensitive Mn-SOD form, whereas the cytosolic supernatant contained the cyanide-sensitive Cu/Zn-SOD form.

Assay for SOD (EC 1.15.1.1) activity. Heart homogenates, mitochondrial pellets and cytosolic supernatants were separately assayed for SOD (7,30). The assay was carried out at a temperature of 25°C in a 3-mL mixture contained in a spectrophotometric cuvette and consisting of 2.5 mL of 0.04 M phosphate buffer (pH 7.45), 0.25 mM xanthine as substrate, 0.3 mM INT as the chromogenic reagent and 0.1 mM ethylenediaminetetraacetic acid as chelator for transition metal ions. The reaction was initiated by addition of 10 milliunits of buttermilk xanthine oxidase (one milliunit of xanthine oxidase, E.C. 1.1. 3.22, converts one nanomole of xanthine to uric acid at pH 7.5 at 25°C). The change in absorbance (0.25–0.45 Units) against a xanthine oxidase blank sample was recorded for 10 min at 550 nm using a Perkin-Elmer 402 spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks, United Kingdom). An aliquot of 200 μL containing 155–455 μg of protein of the heart sample under test was added to both the blank and the experimental cuvettes, and the change in absorbance was recorded for another 10 min. The percent inhibition of heart SOD on the xanthine/oxidase/INT reaction was measured and the activity of the enzyme calculated from calibrated SOD graph, in which one SOD unit exerted 50% inhibition on the xanthine/xanthine oxidase/INT reaction under the conditions of the assay.

TABLE 2

Fatty Acid Composition of Oils[a]

Fatty acid	EPO (wt%)	BOO (wt%)	FGO (wt%)
14:0	0.0	0.1	0.0
16:0	6.2	9.3	22.0
18:0	1.7	2.8	7.0
20:0	0.3	0.6	0.0
22:0	0.0	0.4	0.0
24:0	0.0	0.1	0.0
16:1	0.1	0.4	0.0
18:1	9.9	14.2	37.0
20:1	0.2	4.2	0.0
22:1	0.0	2.7	0.0
24:1	0.0	1.5	0.0
18:2n-6	71.2	38.0	10.0
18:3n-6	9.4	25.0	17.0
18:3n-3	0.3	0.0	0.0

[a]Abbreviations: EPO, evening primrose oil; BOO, borage oil; FGO, fungal oil. Oils were supplied by John E. Sturge Ltd. (Yorkshire, United Kingdom) and ViPONTE Ltd. (Hull, United Kingdom).

Assay for succinate dehydrogenase activity (EC 1.3.99.1). Succinate dehydrogenase activity was assayed at 25°C (31) in 3.0 mL of a mixture containing 0.1 M potassium phosphate buffer, pH 7.8, 0.63 mM potassium ferricyanide, 50 mM sodium succinate, and 160–325 µg of protein of the mitochondrial suspension or 190–325 µg of protein of the cytosolic supernatant. The reaction was followed at 420 nm for 20 min, and the activity of the enzyme was calculated from the change in absorbance and the µM absorption coefficient of potassium ferricyanide, and expressed in µmoles of succinate per min (Units) per mg of protein.

Lipid extraction and fatty acid analysis. Hearts (0.5–0.7 g) were homogenized in 100 vol (wt/vol) of chloroform/methanol (2:1, vol/vol) until a fine suspension was formed, and then extracted overnight at 5°C. The lipid extracts were filtered and then processed according to Folch *et al.* (32). Total lipids were concentrated under a stream of nitrogen and redissolved in 1.0 mL of chloroform and separated by high-performance liquid chromatography (HPLC) on a Varian model 5000 (Varian, Palo Alto, CA). The chromatographic column was a 25 cm × 8 mm prepacked Lichrosorb 5 silica 60 column (Thames Chromatography, Berkshire, United Kingdom), which contained silica of a particle size of 5 µm. The mobile phase solvent system was used isocratically (33). Briefly, Solvent A, 95% acetonitrile, and Solvent B, 5% methanol/85% orthophosphoric acid [methanol/H_3PO_4 (85%) 5:1.5 vol/vol], were delivered to the column at a flow rate of 1 mL/min at a pressure of 26 bar (382 PSI) at 27°C, and detected at 203 nm. Fractions were identified by comparison of their retention times with those of phospholipid standards obtained from Sigma Chemical Co. Choline phospholipids (CPL) and ethanolamine phospholipids (EPL) separated by HPLC were evaporated to dryness under a stream of nitrogen and transmethylated with 5 mL of methanol containing 5% sulfuric acid for 3 h at 70°C, in a nitrogen atmosphere. Methyl esters were recovered by addition of an equal volume of 5% saline and extracted into 3 × 2 mL of petroleum hydrocarbon (b.p. 30–40°C). The extract was dried over anhydrous sodium sulfate, evaporated to dryness and redissolved in 3 mL of Analar grade heptane. Methyl esters were analyzed by gas–liquid chromatography (34).

Statistics. Student's unpaired *t*-test was used to compare and evaluate significant differences between stock diet and experimental results.

RESULTS

Enzymatic activities. The overall (combined) specific activities of SODs (Mn-SOD and Cu/Zn-SOD) in homogenates of hearts from rats fed BOO or FGO diets were significantly higher than the activities in heart homogenates from rats fed the stock diet. Similarly, the respective specific activities of Mn-SOD in heart mitochondrial fractions from borage or fungal oil fed rats were also significantly higher as compared to the activity of heart mitochondrial Mn-SOD from rats fed the stock diet. There was no significant difference between the specific activities of Mn-SOD in heart mitochondria from rats fed BOO or FGO. The activities of Cu/Zn-SOD in cytosolic supernatants from the hearts of rats fed borage, fungal or stock diets were not statistically different. There were no significant changes in the activities of SOD(s) in the homogenate, mitochondrial or cytosolic fractions from hearts of EPO fed rats relative to the respective activities detected in rats fed the stock diet. The overall (combined) specific activity of Mn-SOD and Cu/Zn-SOD detected in heart homogenates from rats fed the FFD was decreased in comparison to the activity in rats fed the stock diet. Similarly, heart mitochondrial fractions from FFD rats exhibited decreased Mn-SOD activity relative to heart mitochondria from stock diet fed rats. No statistical difference was found between the activity of Cu/Zn-SOD in the cytosol of FFD fed rats and that of stock diet fed rats (Table 3).

The activities of the mitochondrial marker succinate dehydrogenase in mitochondria-rich fractions from the hearts of EPO fed rats were 15.15 ± 3.03 µmoles of succinate per min (Units) per mg of protein, from BOO fed rats 13.05 ± 2.40 Units/mg of protein and from FGO fed rats 16.88 ± 2.10 Units/mg of protein. No succinate dehydrogenase activity was found in the cytosolic supernatants. This indicated that mitochondria-rich pellets containing Mn-SOD were effectively separated by centrifugation at 37,000 × *g* from the cytosolic supernatant that contained Cu/Zn-SOD.

Heart CPL fatty acids. A significant increase was detected in the proportion of CPL-linoleic acid (18:2n-6) in the hearts of rats fed EPO relative to the level of 18:2n-6 in the hearts of stock diet fed rats. This resulted in a reduced arachidonic acid to linoleic acid ratio. In contrast, rats fed BOO or FGO showed an increase in heart CPL-arachidonic acid compared to stock diet fed rats. As a consequence, the arachidonic to linoleic acid ratio was increased (Table 4).

Heart EPL fatty acids. In heart tissues of rats fed BOO- and FGO-rich diets, the proportions of EPL-linoleic acid were significantly reduced relative to that in the heart of stock diet fed animals. By contrast, the proportions of heart EPL-arachidonic acid were increased in BOO- and FGO-fed rats. These fatty acid changes in the heart, induced by BOO and FGO diets, resulted in a respective fourfold and eightfold rise in the ratio of EPL-arachidonic to EPL-linoleic acid relative to the ratio in stock diet fed rats (Table 4).

DISCUSSION

The results of the present study suggest that an increase in the ratio of arachidonic to linoleic acid is related to the increase in the activity of mitochondrial Mn-SOD. This is apparent from the effects of the BOO-supplemented diet that resulted in a twofold increase in the activity of the enzyme and a fourfold increase in the EPL-arachidonic to EPL-linoleic acid ratio. Similarly, the FGO-containing diet also resulted in a twofold increase in the activity of Mn-SOD, and an eightfold rise in the EPL-arachidonic to EPL-linoleic acid ratio. In contrast, in EPO fed rats where there was no change in the ratio of EPL-arachidonate to EPL-linoleate, there was no effect on Mn-SOD activity. The increases in the proportion of heart arachidonate

TABLE 3

Effects of Dietary Oils on the Overall Specific Activities of Mn-SOD and Cu/Zn-SOD in Heart Homogenates and Respective Activities in Mitochondria and Cytosol After 29 Days[a]

Diet	Total protein (mg)	Total activity (SOD Units)	Specific activity (SOD Units/mg protein)
Homogenate			
STK	4.05 ± 0.13	9.71 ± 1.4	2.40 ± 0.31
BOO	5.87 ± 0.81[e]	18.36 ± 0.88[b]	3.15 ± 0.20[e]
FGO	4.60 ± 0.17	15.81 ± 1.80[d]	3.43 ± 0.26[d]
EPO	4.55 ± 0.71	11.82 ± 1.10	2.63 ± 0.30
FFD	4.20 ± 0.45	6.16 ± 1.66[f]	1.45 ± 0.25[e]
Mitochondria-rich fraction			
STK	2.38 ± 0.29	2.03 ± 0.29	0.85 ± 0.06
BOO	2.63 ± 0.66	4.93 ± 1.06[e]	1.87 ± 0.06[b]
FGO	2.25 ± 0.44	3.86 ± 0.60[d]	1.73 ± 0.17[b]
EPO	2.05 ± 0.43	1.54 ± 0.17	0.77 ± 0.15
FFD	2.73 ± 0.15	1.18 ± 0.18[e]	0.43 ± 0.09[c]
Cytosolic supernatant			
STK	3.17 ± 0.19	14.65 ± 1.47	4.62 ± 0.35
BOO	2.70 ± 0.13[e]	12.30 ± 0.69	4.57 ± 0.33
FGO	3.67 ± 0.76	16.19 ± 1.04	4.52 ± 1.10
EPO	2.65 ± 0.34	11.54 ± 0.68	4.39 ± 0.43
FFD	3.27 ± 0.15	15.36 ± 2.08	4.69 ± 0.43

[a]Data are reported as means ± SD (n = 3). One superoxide dismutase (SOD) unit exerted 50% inhibition on the assay reaction (0.25 mM xanthine, 10 mUnits xanthine oxidase, 0.3 mM *p*-iodonitrotetrazolium violet, 0.1 mM ethylene diaminetetraacetic acid in 3.0 mL of 0.04 M phosphate buffer, pH 7.45). Abbreviations as in Table 2.
[b–f]*P* values relative to control stock diet: [b]< 0.001; [c]< 0.005; [d]< 0.01; [e]< 0.025; [f]< 0.05.

effected with the BOO and FGO diets probably arose from the high content of γ-linolenic acid. The latter bypasses the rate-limiting Δ6 desaturase step and is readily converted to arachidonic acid (35,36). The finding that Mn-SOD activity is reduced in the heart of rats fed the FFD diet is consistent with the decrease in the activity of adenylate cyclase in the heart of essential fatty acid-deficient rats (37). Interestingly, it has also been reported (38) that dietary n-3 fatty acids decrease the activities of SODs in rat heart. This, when compared to our findings, suggests that n-6 and n-3 fatty acids have differential effects on mitochondrial SOD activity. Changes in the EPL ratio of arachidonic to linoleic acid, brought about by dietary manipulations, have also been observed in the erythrocytes of monkeys with respective changes in the susceptibility of membranes to peroxidative damage (34). One hypothesis that can be advanced to explain the increases in the activities of Mn-SOD observed in the present investigation is that the increased heart membrane arachidonate in rats fed the BOO and FGO diets increases the "peroxidizable index," which may stimulate Mn-SOD activity as a consequence of superoxide radical formation. Mammalian Mn-SOD is a tetrameric metallo-protein consisting of two dimeric subunits (5,39). The enzyme may exhibit allosteric kinetic properties in the presence of reduced or increased quantities of the substrate (superoxide ions). This may explain the 50% decrease in the activity of the enzyme in the mitochondrial fractions from FFD fed rats. It

TABLE 4

Effects of Dietary Oils on Choline Phospholipid and Ethanolamine Phospholipid Fatty Acids of Rat Heart[a]

Diet	LA	AA	AA/LA ratio	Relative % change
Choline phospholipid				
STK	8.8 ± 1.4	18.9 ± 2.0	2.1	100.0
BOO	8.8 ± 1.3	26.2 ± 4.0[f]	2.9	138.1%
FGO	10.2 ± 1.7	28.5 ± 4.3[f]	2.8	133.3%
EPO	16.0 ± 2.2[d]	21.4 ± 2.6	1.3	61.9%
Ethanolamine phospholipids				
STK	2.81 ± 0.60	25.8 ± 3.5	9.2	100.0
BOO	0.80 ± 0.24[d]	32.3 ± 3.5	40.3	438.0%
FGO	0.46 ± 0.17[c]	35.2 ± 3.6[f]	76.5	831.5%
EPO	3.40 ± 0.54	27.6 ± 3.8	8.1	88.0%

[a]Data are means ± SD (n = 3). Abbreviations as in Table 2. LA, linoleic acid (18:2n-6); AA, arachidonic acid (20:4-6).
[c,d,f]*P* values: [c]< 0.005; [d]< 0.01; [f]< 0.05.

would also explain the twofold rise in enzymatic activity with the fourfold or eightfold increases in EPL-arachidinate to EPL-linoleate ratio in the heart of BOO- or FGO-fed rats. A second hypothesis is that the level of membrane arachidonate may modulate the induction/transcription of the Mn-SOD gene (40), possibly *via* the activation of oxidative stress-responsive transcription factors (41).

The dependence of Mn-SOD activity on membrane fatty acid composition may explain the absence or low activity of this enzyme in some leukemia cells (42) of reduced polyunsaturated fatty acid status (43). Similarly, the inactivity of Mn-SOD in Morris hepatoma of high superoxide ion production (44,45) may arise from decreased levels of polyunsaturated fatty acids as a result of peroxidation.

In summary, our studies suggest that mitochondrial Mn-SOD activity is influenced by the fatty acid composition of membrane phospholipids and, in particular, by the proportions of the unsaturated fatty acids linoleic and arachidonic acid.

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Gradient for D-Glucose and Linoleic Acid Uptake Along the Crypt-Villus Axis of Rabbit Jejunal Brush Border Membrane Vesicles

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Glucose uptake into jejunal brush border membrane (BBM) varies along the crypt-villus axis (CVA). In the present study, the question was addressed whether uptake of the essential long-chain fatty acid linoleic acid also varies along the CVA. Using agitation techniques, five jejunal enterocyte fractions were sequentially isolated from female New Zealand white rabbits. A sixth and final fraction of lower-villus/crypt cells was obtained by the scraping of the remaining jejunal mucosa. Cell fraction along the CVA was proven histologically, by noting decreasing alkaline phosphatase activities in sequentially isolated fractions, and by demonstrating [^{3}H-*methyl*]thymidine uptake mainly in the final fraction of the lower villus/crypt cells. BBM vesicles were prepared from the upper-, mid- and lower-villus/crypt enterocyte fractions, using differential centrifugation and divalent ion precipitation. D-Glucose uptake into each fraction showed an Na^+-gradient dependent time-course "overshoot" with linear uptake to 15 s and a subsequent decline to a steady-state plateau. Varying D-glucose concentrations from 50–1000 μM demonstrated saturation kinetics of uptake, with maximal transport rates (V_{max}) and Michaelis affinity constants (K_m) varying between fractions; the K_m and V_{max} were both lowest in the upper-villus fraction. A linear relationship existed between linoleic acid concentration (25–200 μM) and uptake in each fraction. Linoleic acid uptake was equivalent in all fractions when expressed per mg protein, but when expressed in terms of the estimated minimal BBM, vesicle surface area uptake was greater in the upper- than in the lower-villus/crypt fractions. Thus, BBM vesicle uptake of both linoleic acid and glucose vary along the crypt-villus axis of the rabbit jejunum.

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Enterocytes originate in the crypt of the intestinal villus and mature as they advance along the crypt-villus axis (CVA). During this advance, the enterocytes change from cuboidal to columnar cells, and the microvilli change from being broad and relatively sparse in the crypt cells to the narrower and more numerous microvilli of the upper-villus enterocytes (1). The length of the microvilli increases as rat enterocytes advance along the CVA, with lengths of 1.8 mm in microvilli from enterocytes from the upper-villus, as compared to values of 1 and 0.8 mm in mid- and lower-villus enterocytes, respectively (2). As enterocytes advance along the CVA, the intracellular activity of thymidine kinase decreases while the brush border membrane (BBM) activities of alkaline phosphatase (ALP) and invertase (INV) increase (3–13).

Following a meal rich in dietary fats, lipid droplets are seen predominantly in upper-villus enterocytes (14). Autoradiographic studies have demonstrated that the uptake of palmitic acid occurs mainly in the upper-villus fraction of the CVA (15). Similar studies have demonstrated that amino acids and hexose uptake also occur predominantly in the enterocytes of the upper third of the villus (16–19). *In vitro* studies using BBM vesicles prepared from enterocyte fractions harvested from along the CVA have established that there is greater uptake of amino acids and hexoses in the upper than in the lower portions of the CVA (3,5,9,20). Indeed, it has been suggested that a second hexose carrier may appear as the enterocytes migrate along the CVA (3,5,21–23), and that the kinetic properties of carrier-mediated transport vary along the CVA (3,5,9). Taken together, these findings give support to the concept that nutrients are absorbed principally in the upper portion of the villus.

BBM vesicles have been shown to be a useful tool to assess the uptake of linoleic acid (LA) by the small intestine (24,25). However, no published studies are available comparing the uptake of this essential long-chain fatty acid by BBM vesicles obtained from enterocytes originating at different levels of the CVA. Accordingly, the present study was undertaken to test the null hypothesis that there is no gradient in the uptake of LA or D-glucose along the CVA of rabbit jejunum. The results suggest that the null hypothesis is rejected, and that they passive permeability properties for LA, and the maximal transport capacity (V_{max}) and Michaelis affinity constant (K_m) for glucose uptake, are greater for the BBM of enterocytes from the upper as compared to the lower portions of the CVA.

MATERIALS AND METHODS

Preparation of tissue. The guiding principles in the care and use of laboratory animals, approved by the Canadian Federation of Biological Societies (Ottawa, Ontario) and by the Council of the American Physiological Society (Washington, D.C.), were observed in the conduct of this study. Mature female New Zealand white rabbits weighting 1.6–2.2 kg were given *ad libitum* access to standard Purina® (Ralston-Purina, St. Louis, MO) rabbit chow and water until the morning of study. Animals were sacrificed by a lethal injection of sodium thiopental (240 mg/kg) into a marginal ear vein. The abdominal cavity was rapidly opened, and 80 cm of jejunal

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Abbreviations: ALP, alkaline phosphatase; BBM, brush border membrane; CVA, crypt-villus axis; F, fraction of enterocytes along the crypt-villus axis; $FABP_m$, fatty acid binding protein in brush border membrane; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid); INV, invertase; K_m, Michaelis affinity constant of glucose uptake; LA, linoleic acid (18:2n-6); $SGLT_1$, sodium dependent glucose transporter; TK, thymidine kinase; UWL, unstirred water layer; V_{max}, maximal transport capacity of D-glucose uptake.

distal to the ligament of Trietz was removed. This jejunal segment was gently flushed with 100 mL iced saline and was divided into 20-cm segments. The intestinal mesentery was removed, and the jejunal loops were everted over glass rods. The tissue was kept at 4°C.

The subsequent steps of enterocyte harvesting were begun within 20 min of sacrificing the rabbits. The two ends of each jejunal segment were tied after filling the segment with isolation buffer I (154 mM NaCl, 10 mM NaH_2PO_4, 1.5 mM ethylenediaminetetraacetic acid, 0.5 mM glutathione, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.3). Segments of jejunum were individually suspended in beakers containing 20 mL of citrated buffer solution (96 mM NaCl, 1.5 mM KCl, 8.0 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM sodium citrate, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.3), and were gently shaken for 15 min in a water bath heated to 37°C. This initial exposure to citrated buffer solution promoted enterocyte dissociation from the underlying lamina buffer. The initial solutions, labeled Fraction 1 (F1), were pooled in 250-mL polypropylene bottles and stored on ice. After 40 min of agitation, the resultant solutions were labeled Fraction 2 (F2) and pooled in a 250-mL polypropylene bottles for further processing. Next, the jejunal segments were transferred to beakers containing 20 mL of isolation buffer, and were agitated for 10 min. Solutions from this step were pooled as Fraction 3 (F3), and the segments were transferred to a fourth series of beakers containing isolation buffer. After 25 min of agitation, incubation solutions were recovered, labeled Fraction 4 (F4) and pooled in cooled 250-mL polypropylene bottles. Intestinal segments were then transferred to a final series of beakers for 10 min of agitation. The resultant solutions were labeled Fraction 5 (F5) and were pooled in a similar fashion as the previous collected fractions. The intestinal segments were then opened longitudinally, and the remaining mucosa was removed by gently scraping with a microscopic slide. Mucosal scrapings were pooled in cooled 250-mL polypropylene bottles containing 160 mL of isolation bugger, labeled as Fraction 6 (F6) and saved for further processing.

In order to produce more distinct populations of enterocytes from along the CVA, enterocytes from F1, F3 and F5 were not utilized in the study of nutrient uptake along the CVA, and therefore BBM vesicles were not prepared from these fractions. However, all six fractions were utilized in histological and enzyme assay studies to validate the technique of enterocyte fractionation along the CVA.

All subsequent steps were carried out at a temperature of 4°C. Each fraction (F2, F4, F6) was treated separately in a similar fashion. Fractions were centrifuged in a Beckman JA-1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 2,400 × *g* for 5 min. Aliquots were taken from each fraction for protein determination by a modification of the Lowry method using bovine serum albumin (26,27), and for DNA determination by a modification of the Dische reaction of DNA with diphenylamine (28,29). The resultant supernatant was discarded, and the pellets were resuspended in 115 mL of mannitol-Tris buffer (300 mM D-mannitol/10 mM tris-HCl, pH 7.0) in 250-mL polypropylene bottles. Fractions were then homogenized using a Brinkmann Polytron® (Brinkmann Instruments, Westbury, NY) at a setting of "6" for 10 s, and were centrifuged at 600 × *g* in a Beckman JA-14 rotor for 15 min. $CaCl_2$ was added to the supernatants to yield final concentrations of 10 mM $CaCl_2$. The resultant solutions were stirred on ice for 10 min to precipitate subcellular components, and were then centrifuged at 7,800 × *g* for 20 min. The pellets were discarded, and the supernatants were centrifuged at 30,000 × *g* for 20 min. Following this, supernatants were discarded, and the pellets were resuspended in 20 mL of isolation buffer II [300 mM D-mannitol/10 mM *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)(Hepes)-Tris, pH 7.4]. The resultant solutions were homogenized using a Brinkmann Polytron® at a setting of "6" for 15 s, and were then centrifuged at 30,000 × *g* for a final 20 min. The supernatant was discarded, and the pellet was resuspended in 1.0–1.5 mL of Isolation Buffer II. Aliquots of the resultant BBM vesicle-enriched suspension were taken from each fraction for protein determination by a modification of the Lowry method using bovine serum albumin (26,27).

Sections 1 cm in length were taken from jejunal segments at the end of each step in the fractionation process. These specimens were fixed in formaldehyde solution, embedded in paraffin wax, sectioned parallel to the CVA for light microscopy, and stained with hematoxylin and eosin. Photomicrographs were obtained of each specimen to determine villus height in each fraction, as well as the depth of mucosal scraping in F6.

Photomicrographs of histological sections of segments of jejunum demonstrated a progressive decline in the height of villi with each successive fractionation step, with F2 demonstrating removal only of the upper-villus enterocytes and F4 demonstrating removal of the upper-villus and mid-villus enterocytes. Histology of F6 showed virtual absence of villi (data not shown).

Enzyme determinations. Enzyme activity was assayed in both enterocyte homogenates and in the BBM vesicle suspensions. ALP activity was measured by incubating the samples with 2-amino-2-methyl-1-propanol buffer in the presence of $MgCl_2$ at pH 10.25. With the addition of *p*-nitrophenyl phosphate, *p*-nitrophenol was formed as a result of ALP-induced hydrolysis, and the solution changed color. Photometric determination of the resultant color change allowed a quantification of ALP activity in each specimen. INV activity was measured by incubation of the sample with sucrose, with assay by a glucose-specific hexokinase reaction of the glucose formed. Enrichment of enzyme activity in BBM vesicle suspensions as compared to cell homogenates was demonstrated by ratios of 4.1 for INV activity in F2, 6.1 for F4 and 5.1 for F6, and by ratios of ALP activity for 2.6 for F2, 4.0 for F4 and 5.3 for F6.

To indirectly assess thymidine kinase activity, rabbits were injected intravenously with 400 mCi of [^{3}H-*methyl*]thymidine (86 Ci/mmol; Amersham International, Amersham, United Kingdom) in 0.9% NaCl solution, and were sacrificed two hours later. Enterocytes

were isolated from along the CVA, and cell homogenates were prepared from each fraction and transferred to scintillation counting vials (20 mL; Fisher Scientific, Montreal, Quebec, Canada). Scintillation cocktail (Ready-Safe Liquid Scintillation cocktail; Beckman Instruments Inc., Fullerton, CA) was added, and specific activity of [^{3}H-*methyl*]thymidine was determined for each fraction.

Glucose uptake. To determine glucose uptake as a function of incubation time, a solution of 100 mM D-glucose was made up in D-glucose transport buffer (100 mM NaSCN/1 mM $MgCl_2$/2mM $CaCl_2 \cdot 2H_2O$/100 mM D-mannitol/10 mM Hepes-Tris, pH 7.4). D-Glucose in this solution consisted of 1% D-[^{3}H]glucose (4.4 Ci/mmol; Amersham International), which had been dried down under nitrogen in the presence of 99% reagent grade D-glucose. The selected times of incubation were 5, 10, 15, 30 and 45 s and 1, 1.5, 2, 3, 5, 10 and 15 min.

BBM vesicle uptake of glucose was studied at room temperature (20°C). The vesicles had been initially suspended in Na^+-free solution so that a 100 mM inwardly-directed Na^+ gradient was present at the start of the incubation. Incubations of under 1.0 min were carried out by mixing 20 mL of BBM vesicle suspension with 20 mL of D-glucose solution in 17 × 100 mm polystyrene test tubes (Fisher Scientific). For time trials of 1.0 min and longer, incubations of 50 mL of both vesicle suspension and D-glucose solutions were used in similar polystyrene tubes. Reactions were initiated by the mixing of the solutions with a vortex mixer. Uptake was terminated by the use of a stopping buffer (100 mM NaSCN/0.6 mM phlorizin/0.15 M KCl/2 mM Hepes-Tris, pH 7.4). Aliquots of the solution were rapidly filtered through a 0.45-m nitrocellulose filter (Micron Separations, Inc., Westboro, MA) under the vacuum on an Amicon Filtration Manifold VFMI. Filters were prewashed with stopping buffer. The filters were rinsed with 4 mL stopping buffer and were placed in scintillation counting vials. The filters were air-dried for 10 min at 55°C, and 7.0 mL of scintillation cocktail was added to each scintillation vial. Radioactivity associated with the filter was measured using a Beckman LS 9800 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA).

The value of radioactivity used to calculated uptake for each BBM vesicle sample was the net radioactivity after subtraction of the nonspecific binding of radioactivity to the filter, as determined by measuring the uptake of radioactive substrate by the filter in the absence of vesicle suspension. D-glucose uptake trials were carried out in identical fashion for BBM vesicle suspensions derived from enterocytes isolated from F2 (upper-villus), F4 (mid-villus) and F6 (lower-villus/crypt). Each data point represents the average of four duplicate determinations each tested on a minimum of four rabbits.

The time-course study of D-glucose uptake for each fraction showed an "overshoot" phenomenon, with initial linear rates of uptake to 15 s. Accordingly, a 5-s duration of incubation was considered representative of substrate uptake at "initial rate" and was selected for the subsequent studies of D-glucose uptake by the BBM vesicles at varying D-glucose concentrations. BBM vesicle uptake as a function of D-glucose concentration was studied with D-glucose concentrations varied from 50 to 1000 mM at increments of 50 mM. Studies were carried out in an equivalent fashion for BBM vesicle suspensions derived from each fraction. Each data point of glucose uptake represents an average of six duplicate studies from a minimum of four rabbits.

LA uptake. The uptake of LA, 18:2n-6 by jejunal BBM vesicle was studied in a similar fashion as D-glucose uptake: 100 mM LA solutions were suspended in lipid transport buffer (1 mM $MgCl_2$/2 mM $CaCl_2 \cdot 2H_2O$/100 mM D-mannitol/10 mM Hepes-Tris/2 mM taurocholic acid, pH 7.4). LA in this solution consisted of 20% [^{14}C]LA (55.6 mCi/mmol; Amersham International) and 80% reagent grade LA (Sigma Chemical, St. Louis, MO). In the preparation of the LA incubation solution, a 600 mM unlabelled LA stock solution was prepared by dissolving the required amount of LA in 25 mL chloroform. Aliquots were pipetted into tubes, dried under nitrogen and stored at -80°C. On the day of the experimentation, the appropriate amount of unlabelled LA was reconstituted in chloroform with the addition of the required amount of radiolabelled LA. The labeled and unlabelled LA were then dried down under nitrogen and reconstituted in lipid transport buffer.

Studies on BBM vesicle uptake of LA were carried out for 5, 10, 15, 30 and 45 s, and for 1, 1.5, 2, 3, 5, 10 and 15 min. Uptake was arrested using stopping buffer (0.2 mM phloretin/0.15 M KCl/2 mM Hepes-Tris, pH 7.4). To prevent the filters from binding LA, the filters were prewashed with 2.0 mL of cold wash solution (2 mM TC/500 mM LA/0.15 M KCl/2 mM Hepes-Tris). Otherwise, similar filters were used, as described above, for the studies of D-glucose uptake. To determine LA uptake as a function of concentration, as incubation time of 5 s was chosen. LA concentrations of 25–200 mM were used. Each data point of LA uptake represents the average of six duplicate studies from a minimum of four rabbits.

Expression of results and statistical analysis. Since the effective resistance of the intestinal unstirred water layer (UWL) is negligible for isolated cells or BBM vesicle (30), the value of the apparent Michaelis affinity constant was taken as the value of the true constant, K_m. Glucose uptake kinetics were determined by nonlinear regression analysis (Michaelis-Menten) using the Enzfitter® software package (Biosoft; Elsevier-Biosoft, Cambridge, United Kingdom), and using the Systat (SYSTAT Inc., Evanston, IL) program for best-fit curves. The analysis was performed by fitting the observed data points to the Michaelis-Menten equations. As the variance increased with the size of the variable Y (rate of uptake of glucose), data points were weighted in proportion to the reciprocal of the within-concentration estimates of variance. Nonlinear regression analysis was estimated for each treatment. The iterative analysis employed sought to minimize the residual sum of squares between the observed and predicted values. Convergence occurred with 20 iterations using the nonlinear regression modules of Systat. The values of V_{max} and K_m were also assessed by linear regression analysis of the Lineweaver-Burke plot (inverse of glucose uptake *vs.* in-

verse of glucose concentration), and the Eadie-Hofstee plot (glucose uptake *vs.* uptake/glucose concentration).

All values given in the table and figures represent the mean ± standard error. Analysis of variance analysis and Student Newman-Keul's test were used to search for differences between means, with 0.05 taken as the upper limit of significance.

BBM vesicle uptake data are usually normalized to unit protein. When using protein as the denominator, it is assumed that the proportion of glucose carrier to total membrane protein remains constant along the CVA. Morphological data suggests that the number of glucose carriers per unit membrane surface area remains constant from crypt to villus tip (2), but it is unknown whether this is the case for the membrane fatty acid binding protein that is thought to play a role in the uptake of fatty acids, particularly at low concentrations (31). In addition, the BBM surface are becomes amplified as the enterocytes migrate up the CVA (2), so that the appearance of greater uptake of nutrients from the upper rather than the lower portion of the CVA may not necessarily be from a greater true V_{max} of individual glucose carriers per unit BBM surface area from upper-villus cells, but instead may be due to a greater total surface area. For this reason, it may be necessary to use other denominators of uptake. In the time-course studies of D-glucose uptake, the equilibrium values of glucose uptake were lower in F2 than in F6 (Fig. 1); longer periods of incubation confirmed the differences in equilibrium uptake rates of glucose (data not shown). We

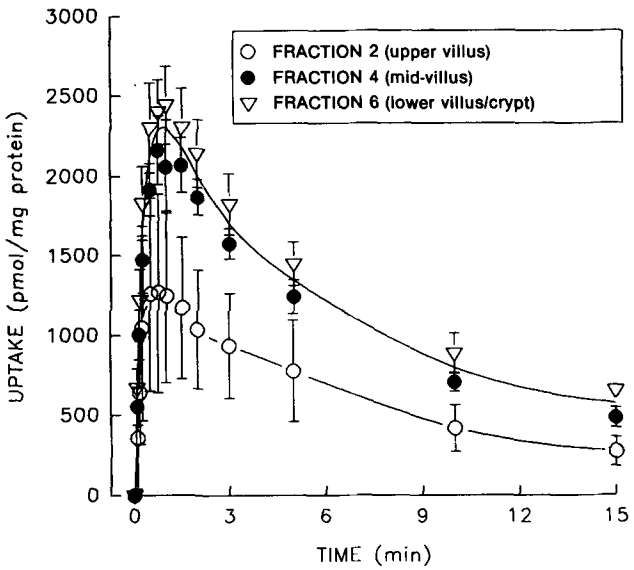

FIG. 1. Uptake of 100 μM D-glucose by brush border membrane (BBM) vesicles, isolated from along the crypt-villus axis, during varying incubation times. BBM vesicles were suspended in 300 mM D-mannitol/10 mM Hepes-Tris, pH 7.4, and uptake of 100 μM D-glucose suspended in 100 mM NaSCN/1 mM $MgCl_2$/2 mM CaCl2 · $2H_2$/100 mM D-mannitol/10 mM Hepes-Tris, pH 7.4, was assayed at 25°C. BBM vesicle uptake of substrate was stopped by the addition of 100 mM NaSCN/0.6 mM phlorizin/0.15 M KCl/2 mM Hepes-Tris, pH 7.4; mean ± SEM.

wished to use a second method of expressing the uptake data on the basis of rate of uptake per glucose carrier or per unit microvillus membrane, but we did not have available to us the molecular biological methods to measure the sodium-dependent glucose transporter ($SGLT_1$), the membrane fatty acid binding protein ($FABP_m$), or the microvillus surface area. We chose to use the equilibrium values for glucose uptake to determine intravesicular volume, and then to calculate the minimum surface area of the BBM vesicles obtained from F2, F4 and F5. We recognize that this approach assumes that the average size of BBM vesicles is similar from F2, F4 and F6. Accepting the limitations of this approach, in some studies glucose and LA uptake were expressed as pmol · unit BBM vesicle surface $area^{-1} \cdot 5\ s^{-1}$, and nmol · unit BBM vesicle surface area · $5\ s^{-1}$, respectively. At equilibrium, the concentrations of D-glucose in the intravesicular space is equal to the D-glucose concentration in the external solution. Since the intravesicular space is substantially smaller than the extravesicular space, the concentration of D-glucose on both sides of the vesicle wall following equilibrium will not be significantly less than the concentration of D-glucose in the external solution at the beginning of the time trial. Thus, at equilibrium, the concentration of D-glucose in the intravesicular space will be very close to this value. The uptake of D-glucose by the vesicular solution at equilibrium was: F2, 275 pmol/mg protein; F4, 487 pmol/mg protein; and F6, 655 pmol/mg protein. Analysis of uptake at equilibrium demonstrated statistically significant differences ($P < 0.05$) between F4 and F6 and between F2 and F6. The calculated intravesicular volumes of the vesicles in F2 is 2.75 mL/mg protein, while that of F4 is 4.87 mL/mg protein and that of F6 is 6.55 mL/mg protein.

The value of the equilibrium uptake has been previously used as a denominator for expression of vesicle uptake. Vinay *et al.* (32) studied lactate transport in basolateral membrane vesicles isolated from both outer medulla and from thick ascending limb segments isolated from the dog kidney. They expressed uptake as a percentage of equilibrium uptake value. We chose to use the values for equilibrium uptake to calculated the surface area of the BBM vesicle, and to express glucose and LA uptake on this basis.

As BBM vesicle form spheres in solution, the total volume of the BBM vesicles can be expressed by the formula $4.3 \cdot \pi R^3$, where R equals the radius of the sphere. The calculated values of R are therefore: F2 0.869 μm, F4 1.052 μm and F6 1.161 μm. The minimum surface area of the BBM vesicle sphere is expressed by the formula $4 \cdot \pi R^2$. The calculated surface area of the BBM vesicle is: F2 9.49 μm^2/mg protein, F4 13.89 μm^2/mg protein and F6 16.93 μm^2/mg protein. The ratios of the surface areas of the BBM vesicles, with the surface area of F2 vesicles assuming the value of 1.0, would be: F2 1.0, F4 1.46 and F6 1.78. Thus, for every unit of surface area in F2 that contains one until of protein, F4 contains 0.68 units and F6 contains 0.56 units. Therefore, the uptake of glucose at initial rate was expressed as pmol · mg $protein^{-1} \cdot s^{-1}$ and pmol · unit BBM surface $area^{-1} \cdot s^{-1}$, and the uptake of LA at initial rate was expressed as

$nmol^{-1} \cdot mg\ protein^{-1} \cdot 5\ s^{-1}$, and nmol · unit BBM vesicle surface area · $5\ s^{-1}$.

RESULTS

Validation of techniques. Numerous methods have been reported in the literature to express the activities of ALP, INV or thymidine kinase (TK). So that our results could be compared to those of others, we chose to express the activities of ALP, INV and TK in several ways. In order to determine the validity of expressing enzyme activity results in terms of the protein or DNA contents of the enterocyte suspensions, the relationship between protein and DNA content of the enterocyte suspensions along the CVA was assessed. The amount of DNA present per cell remains constant as the cell matures along the CVA, but cellular morphology changes along the CVA. Thus, it might be anticipated that the protein content of the enterocyte would also vary along the CVA, with resultant changes in the ratio protein/DNA in each cellular fraction. The lowest ratio of protein/DNA was in F1, highest in F3 and declining in F4–6 (Fig. 2). For this reason, the enzyme activities in the different fractions were expressed on the basis of mg protein as well as mg DNA.

The cumulative amount of protein and DNA obtained from along the CVA in the six fractions was determined, and the percentage of protein or DNA in each fraction relative to the total amount obtained was calculated. The relative position of each fraction on a continuum of protein and DNA content along the CVA could then be determined. The cumulative activity of each enzyme in all six fractions was calculated, and the percentage of total enzyme activity in each fraction was determined. Enzyme activity could thus be analyzed in several ways and expressed in each fraction relative to the protein or DNA content of that fraction, or as percentages of the total amount of enzyme, protein and DNA harvested. Initially collected fractions represented early percentages, with subsequent fractions being added to previous fractions. F2 represented the point at which 6 to 18% of the total protein and DNA had been collected, while F4 was located at the point at which 30 to 45% of protein and DNA had been collected. F6 represented the point at which 100% of protein and DNA had been collected.

ALP activity was lower in enterocyte fractions enriched in mid-villus and lower-villus/crypt cells, as compared to fractions enriched in upper-villus cells (Fig. 3); this was observed regardless of whether ALP activity was expressed relative to the protein or DNA content of the sample, or was expressed as a percentage of the total protein or DNA of the six fractions (F1–F6). INV activity was highest in F1 when expressed as INV/mg protein in the six fractions, or as a percentage of the total pro-

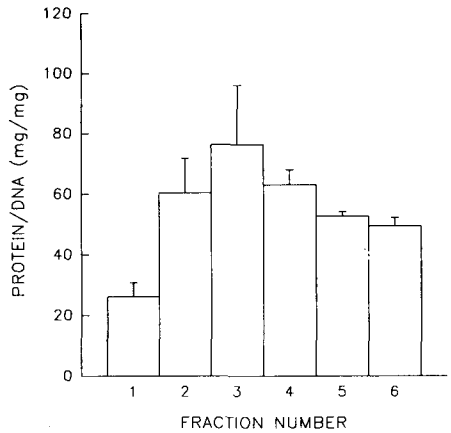

FIG. 2. Ratio of protein content *vs.* DNA content in each fraction along the crypt-villus axis.

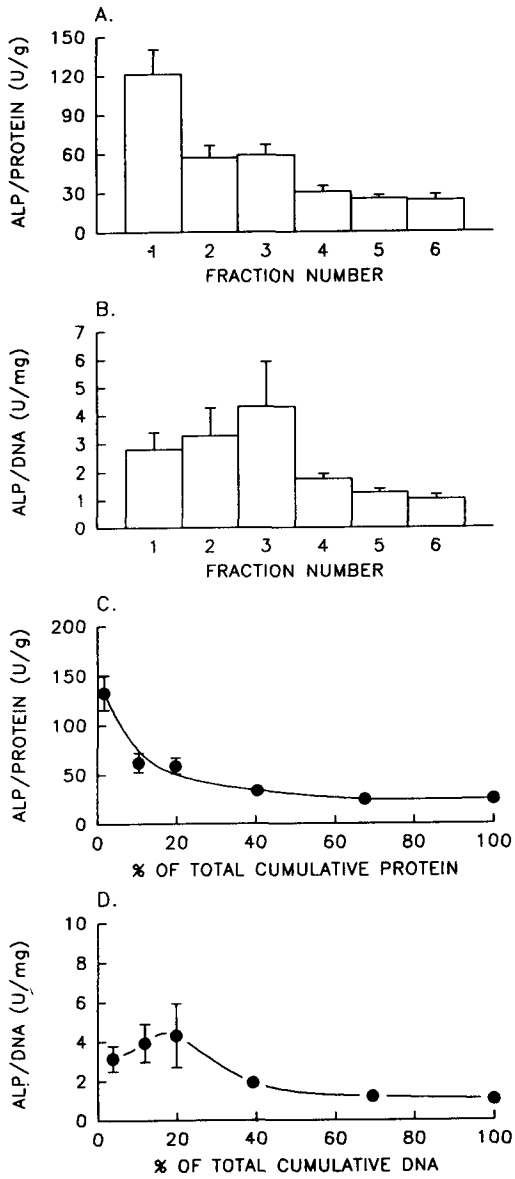

FIG. 3. Specific activity of alkaline phosphatase (ALP) in each enterocyte fraction along the crypt-villus axis. ALP activity in each enterocyte fraction (F1–F6) is expressed per gram protein (A) or milligram DNA (B), or per gram protein *vs.* a percentage of total cumulative protein (C), or per milligram DNA *vs.* a percentage of total cumulative DNA (D); mean ± SEM.

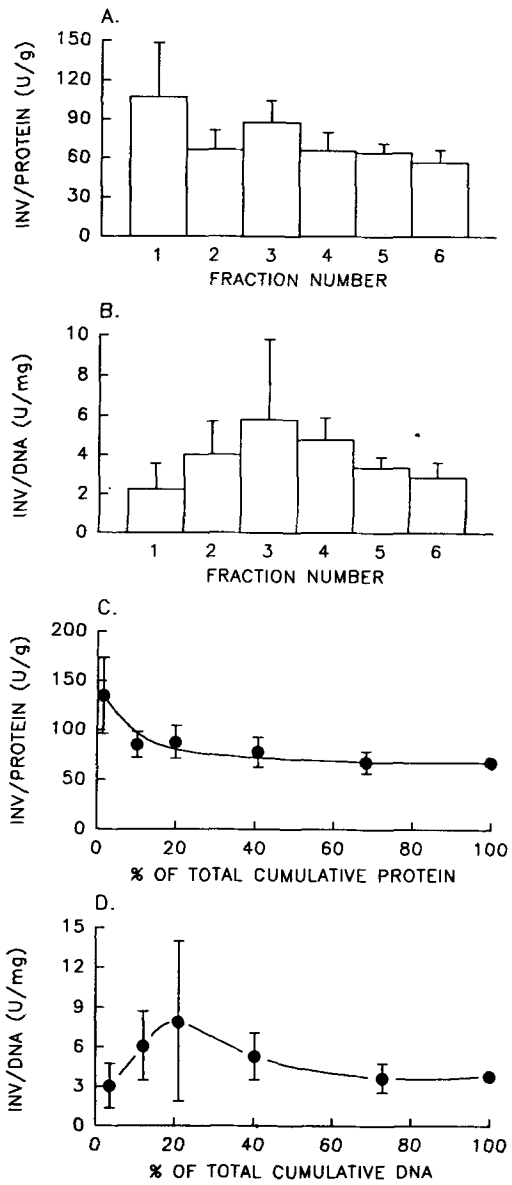

FIG. 4. Specific activity of invertase (INV) in each enterocyte fraction along the crypt-villus axis. INV activity in each enterocyte fraction (F1–F6) is expressed per gram protein (A) or milligram DNA (B), or per gram protein *vs.* a percentage of total cumulative protein (C), or per milligram DNA *vs.* a percentage of total cumulative DNA (D); mean ± SEM.

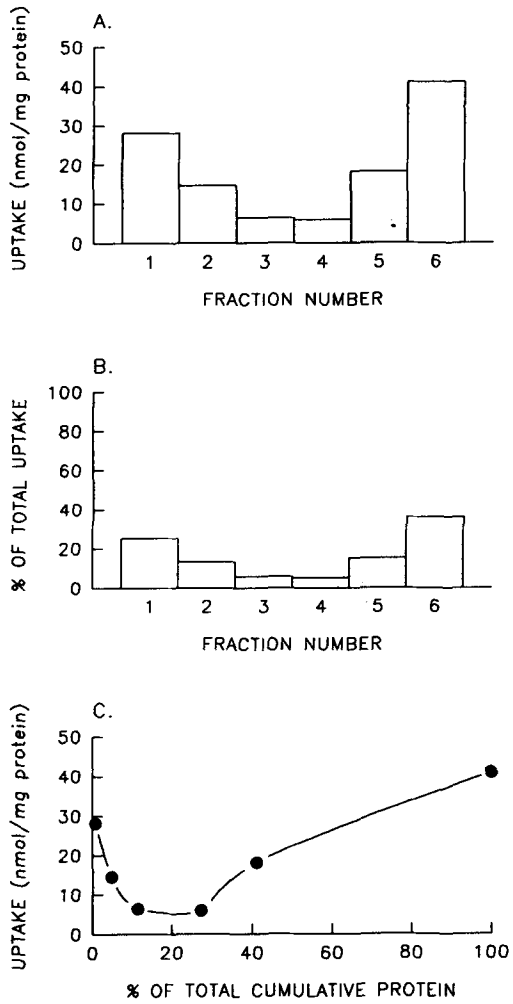

FIG. 5. [^{3}H-*methyl*]thymidine uptake in each enterocyte fraction along the crypt-villus axis. The *in vivo* [^{3}H-*methyl*]thymidine uptake in each enterocyte fraction (F1–F6) is expressed as nmol/mg protein (A), percentage of total uptake (B) or as nmol/mg protein *vs.* percentage of total cumulative protein (C); mean ± SEM.

tein or DNA, and was highest in F3 when expressed as INV/mg DNA (Fig. 4). The uptake of [^{3}H–*methyl*]thymidine was highest in F6 when uptake was expressed as dpm/mg protein or percentage of total uptake in the six fractions. or as dpm/mg protein *vs.* percentage of total protein (Fig. 5).

D-Glucose. The Na^+ dependent uptake of 100 mM D-glucose into BBM vesicles isolated from F2, F4 and F6 showed a time-course "overshoot" with initial rates of uptake being linear up to 15 (Fig. 1). Peak glucose uptake was almost twice as high in fractions enriched in mid-villus and crypt enterocytes (F4 and F6) as compared with the fraction enriched in upper-villus enterocytes (F2). And equilibrium state was achieved within 15 min of onset of incubation of BBM vesicle in D-glucose.

Varying D-glucose concentrations from 50 to 1000 mM and using an incubation of 5 s demonstrated a curvilinear relationship between D-glucose concentration and uptake. The uptake of glucose was similar in all fractions, up to a glucose concentration of 400 mM. At higher concentrations, uptake of glucose was lower in F2 than in F4 or F6. The absolute values of the K_m and V_{max} varied depending upon the method used to estimate the kinetic constants (Table 1, Panel A).

The value of the V_{max} was F2 < F6 < F4 using Systat, Enzfitter with proportional and proportional plus robust weighting, Sigmaplot and Eadie Hofstee plot; the relative ratios were F6 < F2 < F4 for Lineweaver-Burke plot and Enzfitter with simple weighting. When the value of V_{max} was expressed on the basis of the estimated BBM

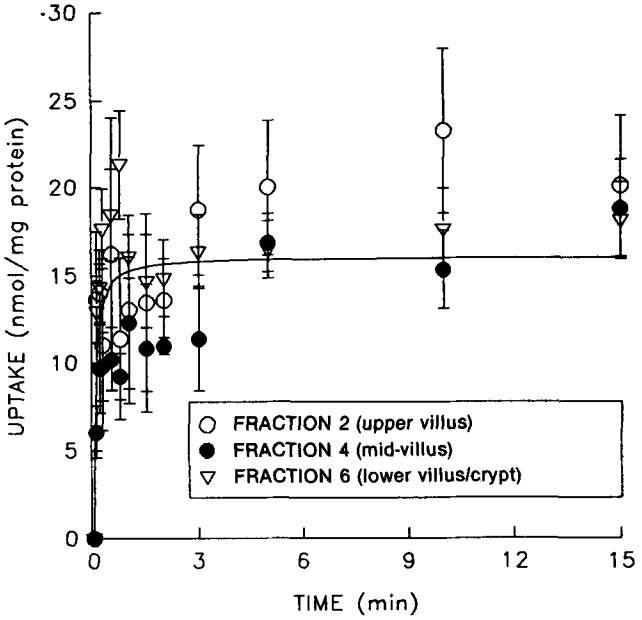

FIG. 6. Time-course of uptake of 100 μM linoleic acid (LA) by brush border membrane (BBM) vesicles isolated from along the crypt-villus axis, with incubation times between 5 s and 15 min. BBM vesicles were suspended in 300 mM D-mannitol/10 mM Hepes-Tris, pH 7.4, an uptake of 100 μM LA suspended in 100 μM LA, 1 mM $MgCl_2$/2mM $CaCl_2 \cdot 2H_2O$/100 mM D-mannitol/10 mM Hepes-Tris/2mM taurocholic acid, pH 7.4, was assayed at 25°C. BBM vesicle uptake of substrate was stopped by the addition of 0.2 mM phloretin/0.15 M KCl/2 mM Hepes-Tris, pH 7.4; mean ± SEM.

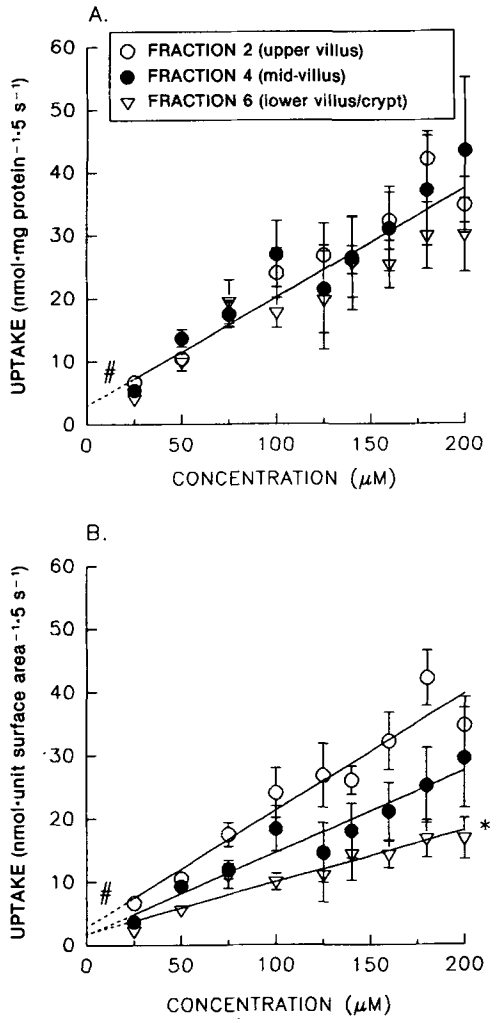

FIG. 7. Effect of varying concentration of linoleic acid (LA) on its uptake by brush border membrane (BBM) vesicles isolated from along the crypt-villus axis as initial rate (5 s). BBM vesicles were suspended in 300 mM D-mannitol 10 mM Hepes-Tris, pH 7.4, and incubated with 25 to 200 μM LA suspended in 1 mM $MgCl_2$/2mM $CaCl_2 \cdot H_2O$/100 mM D-mannitol/10 mM Hepes-Tris/2 mM taurocholic acid, pH 7.4. LA uptake by BBM vesicle suspensions was stopped after 5 s of incubation by the addition of 0.2 mM phloretin/0.15 M KCl/2 mM Hepes-Tris, pH 7.4. Uptake is expressed as nmol · mg protein^{-1} · 5 s^{-1} (A), or as nmol · unit surface area^{-1} · s^{-1} (B), mean ± SEM.

vesicle minimum surface area, the highest values were for the mid-villus (Table 1, Panel B). In all methods of analysis, the value of the K_m was lowest in F2, but the relative values of K_m in F4 and F6 varied between the method of estimation of the kinetic constants.

LA. No overshoot was noted for the rate of uptake of 100 mM LA in F2, F4 and F6, with a plateau of the rate of uptake occurring beyond 5 min of incubation (Fig. 6). With a 5-s incubation and solubilization of LA in 2 mM taurocholic acid, a linear relationship was noted between LA concentration and uptake (nmol/mg protein/5 s) in F2, F4 and F6 (Fig. 1A). The slope of the linear relationship between LA concentration (25–200 mM) and uptake represents the passive permeability coefficient of the BBM. This value was similar for the three fractions and was 0.172 nmol · 5 s^{-1} · mg $protein^{-1}$ · mM^{-1}. The vertical intercept was greater than zero ($P < 0.05$). When LA uptake was expressed on the basis of the calculated BBM vesicle surface area, the passive permeability coefficient was higher for F2 than for F4, and for F4 than for F6 (Fig. 7B), with values of 0.185, 0.130 and 0.079 nmol · 5 s^{-1} · unit surface $area^{-1}$ · mM^{-1} for F2, F4 and F6, respectively. Although the relationship between BBM vesicle LA uptake and concentration was linear, the vertical intercept for each fraction was significantly ($P < 0.05$) greater than zero.

DISCUSSION

Isolation method. Enterocytes can be isolated sequentially from along the CVA, and their origin can be established by determining their profile of enzyme activities (3–10,12,13,33). Cells in the intestinal crypt are rich in enzymes involved in protein synthesis, whereas in the mid- and upper-villus, the major cell functions include the digestion, absorption and metabolism of luminal nutrients. Thus, enterocytes in these fractions of the CVA are rich in enzymes such as ALP and INV, and have decreasing amounts of TK and therefore incorporate less [^{3}H-*methyl*]thymidine (Fig. 5). These findings are simi-

TABLE 1

Kinetic Constants for D-Glucose Uptake Along the Crypt-Villus Axis of the Rabbit Jejunum Using Different Techniques of Data Analysis[a]

Calculation method	A. Uptake per mg protein			B. Uptake per unit surface area		
	Upper-villus F2	Mid-villus F4	Lower-villus F6	Upper-villus F2	Mid-villus F4	Lower-villus F6
Michaelis-Menton						
Systat						
(proportional weighting)						
V_{max}[b]	1448 ± 79	4569 ± 191	3122 ± 538	1448 ± 79	3111 ± 134	1711 ± 292[d]
K_m	90 ± 22	559 ± 51[c]	655 ± 20	90 ± 22	560 ± 52[c]	628 ± 196[c]
Sigmaplot						
(simple weighting)						
V_{max}	1718 ± 272	5441 ± 591[c]	4131 ± 1059[c]	1718 ± 272	3700 ± 402[c]	2313 ± 593[d]
K_m	134 ± 13	918 ± 116	1098 ± 526[c]	134 ± 13	918 ± 116	1098 ± 526[c]
Enzfitter						
(simple weighting)						
V_{max}	1720 ± 272	5895 ± 491[c]	2438 ± 391[d]	1720 ± 272	4009 ± 334[c]	1365 ± 220[d]
K_m	135 ± 13	833 ± 100[c]	420 ± 96[c,d]	135 ± 13	833 ± 100[c]	420 ± 96[c,d]
(proportional weighting)						
V_{max}	1696 ± 235	8657 ± 1581[c]	3221 ± 317[d]	1696 ± 235	5880 ± 1066[c]	1797 ± 181[d]
K_m	219 ± 61	1633 ± 503[c]	943 ± 283	219 ± 61	1631 ± 500[c]	935 ± 278
(proportional + robust weighting)						
V_{max}	2025 ± 372	7100 ± 711[c]	4414 ± 1109[d]	2025 ± 372	4841 ± 486[c]	2459 ± 648[d]
K_m	148 ± 43	1211 ± 289	1352 ± 411[c]	148 ± 43	1213 ± 291	1345 ± 433[c]
Lineweaver-Burke						
Sigmaplot						
(simple weighting)						
V_{max}	2188 ± 327	6092 ± 1376[c]	3824 ± 1350	2188 ± 327	4147 ± 961	2127 ± 739
K_m	388 ± 145	1079 ± 296	879 ± 268	388 ± 145	1083 ± 305	872 ± 261
Eadie-Hoffstee						
Sigmaplot						
V_{max}	1522 ± 266	4228 ± 902[c]	2855 ± 975	1522 ± 266	2874 ± 613	1599 ± 547
K_m	81 ± 17	556 ± 177[c]	412 ± 175	81 ± 17	556 ± 177[c]	412 ± 176

[a]The uptake of D-glucose by brush border membrane vesicles prepared from F2, F4 and F6 (fraction of the enterocytes along the crypt-villus axis) were determined at initial rate (5 s) as described in the Materials and Methods section. Uptake is expressed as pmol/mg protein · 5 s (A), or as pmol · unit surface area · 5 s (B), mean ± SEM. Abbreviations: V_{max}, maximum transport capacity of D-glucose uptake; k_m, Michaelis affinity constant of glucose uptake.

[b]Mean ± SEM; V_{max}, pmol · mg protein · 5 s or pmol · unit surface area · 5 s; K_m mM.

[c]$P < 0.05$, F4 *vs.* F2, F6 *vs.* F2.

[d]$P < 0.05$, F6 *vs.* F4.

lar to those described by others. For example, using a technique that involved assaying enzyme activity in segments of intestinal wall sectioned horizontally from along the CVA in adult Wistar rats, Nördstrom *et al.* (11) demonstrated highest values for ALP from the upper quarter of the villus, with values declining toward the base. INV was also low closer to the villus base, but persisted over a greater length of the villus. Sundaram *et al.* (12) reported ALP as a percentage of the values in the villus tip component. This was the highest value, and the activity of INV declined moving from the villus tip to the base. Freeman *et al.* (5) also found the highest ALP to be in the villus tip fraction, with values declining progressively toward the mid-portion of the villus, with little further change between that point and the base of the villus. ALP and INV activities were expressed as mmol · min^{-1} · mg $protein^{-1}$, and were plotted against the percent of isolated cells. There was a progressive and similar decline in activity moving from the initial 20% to the final 100% of isolated cells. Morrill *et al.* (10) plotted sucrase/isomaltase activity relative to protein or DNA content, as well as plotting enzyme activity *vs.* the percentage of total protein or the percentage of total DNA. They found that the highest values appeared in the mid-portion of the CVA when expressed as units/mg protein *vs.* percentage of total protein, whereas sucrase/isomaltase activity was highest in the villus tip when expressed as units/mg DNA or *vs.* percentage of total DNA. This was presumably because the ratio of protein/DNA declined from the villus tip to the crypt. We confirm this finding (Fig. 4). Hartman *et al.* (6) expressed sucrase activity as units/gram protein and determined that the highest activity was in the mid-villus fraction, with lower values at the villus tip and lower-villus/crypt. Dudeja *et al.* (3) demonstrated highest values of sucrase when expressed on the basis of protein, in villus tip cells as compared with low values in lower-villus and intermediate values in the mid-villus. Thus, the pattern of distribution of ALP and INV activity varies from method to method, and with the manner

used to express the results, but activities are generally higher in the upper than in the lower portion of the villus (Figs. 3 and 4).

Regardless of the method of expression of TK used in previous studies (3,5,9), greater than 60% of activity appears in the lower-villus fraction. In our study, the highest uptake of [^{3}H-*methyl*]thymidine was found in the basal portion of the villus (Fig. 5). Thus, F2 represents the upper portion of the villus while F6 represents the basal portion of the villus and the crypt cells, and F4 represents the mid-portion of the villus. It is critical to note, however, that the pattern of change reported in our study and those reported by others depend upon the method used to express the result. It is likely that our upper-, mid- and lower-villus fractions may have been different from those reported by other authors.

Glucose uptake. Glucose entry into enterocytes occurs as the result of Na^+ dependent coupling to a cotransporter in the BBM (3,5,9,21,22). In this study, glucose transport in the presence of an inwardly-directed Na^+ gradient ($NaSCN_{out}$ = 100 mM; $NaSCN_{in}$ = 0) at a D-glucose concentration of 100 mM demonstrated the expected time-course "overshoot" phenomenon in BBM vesicle derived from all three fractions (Fig. 1). Incubation of BBM vesicle with glucose in concentrations between 50 and 1000 mM demonstrated saturation kinetics (Table 1). Although the intestinal $SGLT_1$ has been isolated and cloned (34–38), the kinetic characterization of intestinal glucose transport remains focused on the estimation of the values of the V_{max}, the K_m and the passive permeability coefficient. Theoretical and experimental considerations have demonstrated the importance of correction for the intestinal UWL resistance (39). Using isolated BBM vesicles minimizes the contribution of passive permeation and the effective resistance of the UWL (30), but there remains considerable variability in the magnitude of the estimates of V_{max} and K_m, dependent on the methods used to estimate them (30).

Linear transformations on the Michaelis-Menten equation may not always produce valid estimates of V_{max} and K_m, particularly in the presence of an UWL and when the magnitude of the passive component is not taken into account (40–43). The nonlinear methods of estimating V_{max} or K_m does not change, or may obscure the demonstration of differences where such differences do, in fact, exist. In addition, differences may result from the use of parametric *vs.* nonparametric methods to estimate the magnitude of V_{max} and K_m (45,46). Furthermore, the lack of consensus for the optimal method of estimating these kinetic constants results in difficulty comparing experimental results obtained in one laboratory using one method with those obtained in another laboratory using a second method. Accordingly, we chose to analyze the kinetic data for glucose uptake, V_{max} and K_m, with several methods of analysis.

Utilizing BBM vesicles harvested from along the CVA using fractionation techniques, other investigators have established that a gradient of glucose uptake exists along the CVA of the proximal small intestine. Meddings *et al.* (9) demonstrated in fasted young male New Zealand white rabbits that the highest rates of transport of glucose occurred in BBM vesicles isolated from upper- and mid-villus cells. These fractions are approximately comparable to our F2 and F4. They recorded a K_m for glucose uptake by BBM vesicle obtained from enterocytes isolated from the lower-villus/crypt, F6 (Table 1). Also, Meddings *et al.* (9) used the Systat program to obtain estimates of V_{max} that were highest in cells from the upper-villus, intermediate in cells from the mid-villus and lowest from the lower-villus (47.8, 40.0 and 7.7 nmol · mg protein^{-1} · min^{-1}, respectively). These findings have been supported by autoradiography in *in vivo* glucose studies by Kinter and Wilson (18) and Fedorak *et al.* (47), which demonstrated that most glucose uptake occurred in the enterocytes of the upper-villus.

Dudeja *et al.* (3) used the Lineweaver-Burke method to estimate the value of V_{max} and K_m. They demonstrated, in fasted adult male Lewis rats, a K_m for D-glucose transport of 121 mM for BBM vesicles derived from the villus tip cells, a value of 164 mM for BBM vesicles derived from the mid-villus level and 44 mM for BBM vesicles derived from crypt cells. Values for V_{max} of 904, 1296 and 498 pmol · mg protein^{-1} · min^{-1} from higher *vs.* lower portions of the villus, and K_m values of 482 and 14 mM from the same positions along the CVA. Thus, in these three studies using male rabbit and rat BBM vesicle, V_{max} and K_m were highest in upper- and mid-villus enterocytes, when uptake was expressed on the basis of mg protein and when different methods were used to estimate the values of V_{max} and K_m. This was opposite to the gradient described in this study when our data were expressed as pmol · mg protein^{-1} · 5 s^{-1} (Table 1).

In the present study with each of the methods used to estimate the value of V_{max}, the lowest value was obtained in F2. Similarly, the value for K_m was lowest in F2 for each method of analysis. The relative ratio of the V_{max} or K_m between F2, F4 and F6 varied depending upon the method of analysis. For example, for V_{max} the relative values were F2 < F6 < F4 using Systat, Enzfitter with proportional and proportional plus robust weighting, Sigmaplot and Eadie-Hofstee plot. By contrast, the relative ratios were F6 < F2 < F4 for Lineweaver-Burke plot and Enzfitter with simple weighting. Differences were also noted in the relative values of the K_m depending upon the method used for these estimations. The K_m values were F2 < F6 < F4 with Systat, Enzfitter simple and proportional weighting groups as well as the Eadie-Hofstee and Lineweaver-Burke plots. In contrast, the ratio was F2 < F4 < F6 for Systat, Sigmaplot and Enzfitter proportional and robust analyses. Thus, since it is not possible to determine which of these is the "correct" value for estimation of V_{max} and K_m, it is suggested that Systat and at least one other program be utilized. Regardless of which methods of kinetic analysis were used, the values of the V_{max} and K_m were either lowest in F2 as compared with F4 and F6, or the value of V_{max} was unchanged and the value of the K_m more than doubled between F2 and F6. Thus, different methods of kinetic analysis are unlikely to have been the explanation for our inability to confirm

the qualitative nature of the previously-reported gradient of kinetic constants for glucose uptake along the CVA of the jejunum.

These differences in the relative values of V_{max} and K_m between the villus fractions may have been due to a different population of enterocytes harvested from along the CVA. For example, intestinal scraping to obtain crypt cells was not employed in the studies of Dudeja *et al.* (3) or Freeman *et al.* (5). Our upper-villus fraction used to determine glucose uptake (F2) may have had different transport properties than the very most distal cells sometimes used by others. Other differences between these and out studies included the age or gender of the animals used, and variations in their recent intake of food. In this study, mature female rabbits were used with weights in the range of 1.6 to 2.2 kg, as compared to younger male rabbits with a weight range of 0.5 to 0.75 kg used in the studies of Meddings *et al.* (9). In a male Wistar rat model, Na^+-dependent D-glucose transport varied with age, with young animals having a greater capacity for Na^+-dependent D-glucose transport (48). The male rats studied by Dudeja *et al.* (3) were fasted for 18 h prior to sacrifice. Rabbits studied by Meddings *et al.* (9) underwent overnight fasts before being sacrificed. Rabbits used in this study were allowed *ad libitum* access to food and water up until time of sacrifice. Prior experiments have demonstrated that changes occur in the BBM as a result of malnutrition that may influence transport characteristics (49,50). It is unknown whether simple fasting overnight without previous protein–energy malnutrition or dietary carbohydrate changes alters the relative values of V_{max} and K_m for glucose uptake along the CVA. It is also unknown what effect gender differences might have on the expression of sugar or lipid transport along the CVA.

The mechanism of these changes in glucose uptake kinetics along the CVA is unclear. Meddings *et al.* (9) postulated that glucose transport is influenced by BBM fluidity: these workers noted that V_{max} is higher across the rigid BBM of the mid- and upper-villus enterocytes, as compared to the more fluid BBM of the crypt cells. Dudeja *et al.* (3) did not demonstrate an association between membrane fluidity and glucose uptake: despite being less fluid than their control counterparts, upper-villus BBM vesicles from diabetic rats had similar glucose uptake rates.

Several studies have postulated that more than one D-glucose carrier is present in the BBM. Harig *et al.* (21) using BBM vesicles prepared from human jejunum and ileum, detected a single high-affinity, low-capacity transporter along the entire small intestine, plus a second low-affinity, high-capacity carrier limited to the proximal jejunum. Kaunitz and Wright (22), using bovine and rabbit small intestine preparations, similarly demonstrated two Na^+-dependent saturable transporter systems: a high-capacity, low-affinity system and a second system with a lower capacity and higher affinity. Malo (23) was able to identify a low-affinity high-capacity system as well as a high-affinity low-capacity system in studies of human fetal small intestine, but not when using vesicles obtained from human adult tissue donors (45,46). Both Freeman *et al.* (5) and Dudeja *et al.* (3) have postulated that at least two distinct Na^+-dependent D-glucose transporters are present in the BBM along the CVA. This possibility has been raised previously, based on theoretical considerations (51), and the variations in glucose uptake along the CVA may reflect different proportions of the two postulated carriers. each of which has different kinetic properties. However, molecular biological methods have not suggested the presence of multiple intestinal carriers (34), and Enzfitter analysis of nonparametrically-assessed uptake rates in adult animals is compatible with a single glucose carrier (45,46) (Table 1). Thus, variations in the ratio of two postulated hexose carriers would not likely explain the qualitative differences in the values of the V_{max} and K_m reported for different CVA fractions in our study, as compared with findings by other authors.

LA uptake. The intestinal uptake of lipids has been considered to be by a process of passive diffusion (52). The UWL and the BBM represent the major barriers to the movement of lipids into the enterocyte. The UWL has been considered the rate-limiting factor in the mucosal uptake of fatty acids (53). An $FABP_m$ of rat jejunum has been identified, and it has been suggested that the uptake of fatty acids by the BBM may be mediated in part by $FABP_m$ (31). In a study of LA transport by rabbit BBM vesicles, Ling *et al.* (25) demonstrated an overshoot phenomenon of uptake as a function of time. We were unable to confirm the presence of an overshoot either in this study (Fig. 6) or in a previous study using a homogenate of enterocytes obtained from along the entire CVA of mature rabbit Jejunum (24). This in contrast to the pressure of such an overshoot for the uptake of D-glucose (Fig. 1), which suggests the presence of a sodium-dependent uptake step (24). A linear relationship was found between LA concentrations of 25–200 mM and uptake at the initial rate of 5 s. This is consistent with a process of passive uptake, but does not exclude the possibility of the presence or importance of $FABP_m$. This point is particularly relevant because the Y-axis intercept of the relationship between LA concentration and uptake was different from zero (Fig. 7).

Autoradiographic studies with [^{3}H]palmitic acid have demonstrated higher uptake in villus tip enterocytes than in enterocytes found in the crypt (15). It is possible that the *in vivo* uptake of fatty acids varies along the CVA as a result of changes in the effective resistance of the UWL (54). The fluidity of the BBM falls from crypt to villus tip (9), so that these differences in lipid uptake along the CVA cannot be explained by variations in BBM fluidity. There is no gradient of LA uptake in F2, F4 or F6 when expressed as nmol · mg protein^{-1} · 5 s^{-1} (Fig. 7A) but when LA uptake is expressed as nmol · unit surface area^{-1} · 5 s^{-1}, the permeability coefficient was greater for F2 than for F4 or F6 (Fig. 7B). Thus, lipid uptake *in vivo* may be greater in the upper portion of the villus because of variations in the dimension of the UWL, greater membrane surface area of the enterocytes from the upper than from the lower portion of the CVA, greater passive permeability coefficients of BBM isolated from enterocytes near the upper portion of the vil-

lus or differences along the CVA in the contribution of the $FABP_m$.

The intestine is capable of adaptation, with variations in nutrient uptake occurring, with aging (48), dietary lipid changes (55), diabetes mellitus (3,16,47,56), bowel resection (57) or ethanol ingestion (58,59). In rats rendered hyperglycemic by the injection of streptozotocin, glucose uptake in the ileum is increased by "recruitment" of enterocytes from more distal locations along the CVA (47). It remains controversial whether this is the mechanism of increased glucose uptake in the jejunum in diabetes (3,16). It also remains to be established whether altered uptake of lipids in these models of intestinal adaptation results from changes in the passive permeability coefficients of enterocytes on the upper portion of the villus, or from changes in all of the BBM from enterocytes from along the CVA.

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The Effect of Dietary Fat Level and Quality on Plasma Lipoprotein Lipids and Plasma Fatty Acids in Normocholesterolemic Subjects

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This study examined the effect on the plasma lipids and plasma phospholipid and cholesteryl ester fatty acids of changing from a typical western diet to a very low fat (VLF) vegetarian diet containing one egg/day. The effect of the addition of saturated, monounsaturated or polyunsaturated fat (PUFA) to the VLF diet was also examined. Three groups of 10 subjects (6 women, 4 men) were fed the VLF diet (10% energy as fat) for two weeks, and then in the next two weeks the dietary fat in each group was increased by 10% energy/week using butter, olive oil or safflower oil. The fat replaced dietary carbohydrate. The VLF diet reduced both the low density lipoprotein (LDL)- and high density lipoprotein (HDL)-cholesterol levels; addition of the monounsaturated fats and PUFA increased the HDL-cholesterol levels, whereas butter increased the cholesterol levels in both the LDL- and HDL-fractions. The VLF diet led to significant reductions in the proportion of linoleic acid (18:2ω6) and eicosapentaenoic acid (20:5ω3) and to increases in palmitoleic (16:1), eicosatrienoic (20:3ω6) and arachidonic acids (20:4ω6) in both phospholipids and cholesteryl esters. Addition of butter reversed the changes seen on the VLF diet, with the exception of 16:1, which remained elevated. Addition of olive oil resulted in a significant rise in the proportion of 18:1 and significant decreases in all ω3 PUFA except 22:6 compared with the usual diet. The addition of safflower oil resulted in significant increases in 18:2 and 20:4ω6 and significant decreases in 18:1, 20:5ω3 and 22:5ω3. These results indicate that the reduction of saturated fat content of the diet (<6% dietary energy), either by reducing the total fat content of the diet or by exchanging saturated fat with unsaturated fat, reduced the total plasma cholesterol levels by approximately 12% in normocholesterolemic subjects. Although the VLF vegetarian diet reduced both LDL- and HDL-cholesterol levels, the long-term effects of VLF diets are unlikely to be deleterious since populations which habitually consume these diets have low rates of coronary heart disease. The addition of safflower oil or olive oil to a VLF diet produced favorable changes in the lipoprotein lipid profile compared with the addition of butter. The VLF diets and diets rich in butter, olive oil or safflower oil had different effects on the 20 carbon eicosanoid precursor fatty acids in the plasma. This suggests that advice on plasma lipid lowering should also take into account the effect of the diet on the fatty acid profile of the plasma lipids.
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It is generally accepted that high plasma cholesterol levels are associated with an increased risk of occlusive vascular disease (1). Since it is possible to manipulate the plasma cholesterol level in most people by dietary means, numerous studies have been conducted examining the effect of different foods and dietary treatments on plasma cholesterol levels. In particular, there has been a focus of research on the relationship between dietary fats and cholesterol on plasma cholesterol levels. The results have led to recommendations to the populations in many western countries to reduce the total fat content of their diets, and particularly the amount of saturated fat. Both monounsaturated fat and polyunsaturated (PUFA) are considered beneficial, although in Australia recent advice on dietary modification has suggested that, where possible, saturated fats should be replaced by monounsaturated fats (2). There is also an increasing awareness of the importance of the two different families of PUFA (ω6 and ω3), and national committees in a number of countries have recommended that the level of the ω3 PUFA in the diet should be increased (2).

Much of the research on reducing plasma lipid levels has focused on the quality (fatty acid type) of the fats and less on the total fat content of the diet. Generally, the recommendations have suggested that the total fat intake should be reduced to about 30% of energy. There is concern about reducing the fat level below this for a number of reasons, including difficulty in achieving compliance to low-fat diets and the adverse effect of low-fat diets on high density lipoprotein (HDL)-cholesterol levels (3).

The purpose of the present investigation was to examine the effect of both the quantity and the quality of the fat on plasma lipid levels and plasma fatty acids. The study was designed so that all subjects started from the same point by eating a very low-fat (VLF) vegetarian diet (<10% energy) containing one egg daily for 2 wk and then increasing the fat content of their diet over a 2-wk period to 30% energy from fat using one of the following three fat sources: butter (saturated fat), olive oil (monounsaturated fat) or safflower oil (polyunsaturated fat).

METHODS

Subjects. Thirty free-living subjects (18 women, 15 premenopausal and 3 postmenopausal, and 12 men) participated in the study. Mean body mass index for the women was 22.4 ± 2.6 kg/m^2 (mean ± SD) and for the men was 24.0 ± 1.6 kg/m^2. The subjects were randomly assigned to three groups of 10 subjects (6 women, 4 men), who were designated to the butter group, the olive oil group or the safflower oil group. In the butter group, the mean age was 35.2 ± 14.6 yr, the mean weight was 67.3 ± 12.1 kg and the mean body mass index 23.1 ± 2.3 kg/m^2. In the olive oil group, the mean age was 35.3 ± 13.7 yr, the mean weight was 68.0 ± 10.8 kg and the mean body mass index 23.2 ± 1.9 kg/m^2. In the safflower oil group, the mean age was 39.3 ± 13.3 yr, the mean weight was 66.0 ± 12.0 kg and the mean body mass index was 22.9 ± 3.0 kg/m^2. There was no

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Abbreviations: CE, cholesteryl esters; LDL, low density lipoproteins; HDL, high density lipoproteins; PL, phospholipids; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoproteins; VLF, very low fat.

significant difference in age, weight or body mass index among the three groups (analysis of variance).

Design of the study. The protocol was approved by the Ethics Committee of Deakin University (Geelong, Victoria, Australia). All subjects gave their written voluntary consent before participating and were free to withdraw from the study at any stage. The subjects were each provided with digital kitchen scales and asked to weigh and record all food and liquid intake for the full five weeks of the study. The subjects reported to the University on a weekly basis for dietary counseling and supervision. During this appointment, fasting blood samples were taken, the previous week's food diary was collected and checked, and subjects were instructed of any changes to be made to their diet for the coming week. The subjects prepared most of their meals at home and were closely monitored each week through seven-day weighed food records. They were asked to maintain their usual pattern of physical activity. Any sign of illness, medications used and any deviations to the diet were reported weekly to the dietitian. Several subjects who started the diets could not complete the full study and their results were not included; the reasons for failure to complete included personal problems, lack of motivation and inability to maintain weight.

Each study lasted for five weeks with each subject acting as his or her own control using a protocol we have previously devised (4,5). In our experience and that of others, serum lipid levels stabilize within 7–14 d following a dietary change (4–6). Volunteers followed their habitual diet, which was not vegetarian, during the one-week baseline period. In the second and third weeks they consumed a VLF vegetarian diet with the addition of one egg per day. The addition of the egg to the vegetarian diet was to provide a constant cholesterol intake in the low to normal range typically found in the Australian diet. Because of the low energy density and high bulk of the diet, a carbohydrate supplement (a glucose polymer) was provided to maintain energy intake and to prevent weight loss (Polycose®; Ross Laboratories, Columbus, OH). This supplement was adjusted on an individual basis to be equivalent to 20% energy and was provided as two drinks daily. We have previously used this supplement to maintain energy intakes in similar studies on VLF diets (5). In the fourth and fifth weeks, the subjects continued to eat one egg/day; however, the fat content of the diet was increased in a stepwise fashion to 20% energy in week 4 and 30% energy in week 5 by substituting butter, olive oil or safflower oil, for either one-half of the carbohydrate supplement (week 4) or all of it (week 5). This ensured that there were no major changes in the composition of the diet over the weeks 2 to 5, except for substitution of fat for carbohydrate supplement. The average quantities of added fats (butter, olive oil, safflower oil) were 22 and 44 g/d in weeks 4 and 5, respectively.

A research dietitian instructed each subject individually to eat a wide variety of suitable foods to ensure a nutritionally adequate diet and to maximize the palatability of the diet. Allowable foods included skim milk and other nonfat dairy products, grains and cereal products, such as bread and pasta, fruit and vegetables, except soybeans, olives and avocados. Jam, soft drinks and nonfat confectioneries were also allowed. Fats, oils, nuts, meat, fish, chicken and commercial foods with added fat were excluded from the diet. An extensive list of nonfat vegetarian recipes and some preprepared low-fat vegetarian meals were supplied to provide variety and convenience (Spotless Catering; North Geelong, Victoria, Australia).

Dietary composition was calculated using *Microdiet* (Salford University, Surrey, United Kingdom) and SODA (Computer Models, Cottesloe, W.A., Australia) software packages, based on United Kingdom and Australian data, respectively, but with our own data incorporated for cholesterol content of Australian eggs (389 mg/100 g edible egg) (7). Body weights were measured to the nearest 0.1 kg on a weekly basis.

These studies were conducted over a 2-yr period (July 1988–June 1990), and subjects were enrolled into the diet groups in random order to reduce any possible effects of time on the results. In most cases, there were 4–6 subjects on diets at any one time.

Lipoprotein lipid analysis. Cholesterol and triglyceride levels in fasting plasma were measured enzymatically after enzymatic hydrolysis on a centrifugal analyzer (Centrifichem System 500; Interscience Group, Moorabin, Victoria, Australia) using commercially available kits (Cholesterol Monotest; Triglyceride GPO-PAP; Boehringer, Mannheim, Germany). In our laboratory, the normal range for cholesterol concentrations in fasting plasma is 3.5–5.5 mmol/L and for triglycerides 0.5–2.0 mmol/L; quality control samples were routinely analyzed with each batch of samples examined. Very low density lipoproteins (VLDL) were separated by ultracentrifugation of plasma at 436,000 × *g* for 3 h (Beckman TK 100 ultracentrifuge; Beckman Australia, Mt. Waverley, Victoria, Australia). HDL were separated within two hours of blood collection by precipitation with 15% polyethyleneglycol 6000 (BDH Chemicals, Kilsyth, Victoria, Australia). Lipids in low density lipoproteins (LDL) were calculated by difference between whole plasma and the sum of VLDL- plus HDL-cholesterol concentrations (4). Triglyceride concentrations were measured in whole plasma and VLDL fractions only.

Plasma cholesteryl ester (CE) and phospholipid (PL) fatty acid analyses. Lipids were extracted from plasma by standard techniques, and the CE and PL fractions were separated from the other plasma lipids by thin-layer chromatography (8). The methyl esters were formed by saponification followed by transesterification in BF_3 in methanol, and these were separated by capillary gas–liquid chromatography using a 50 m by 0.32 mm i.d. fused silica column CP Sil 88 column (Chrompak, Middelburg, The Netherlands) as described previously (8).

Statistical analyses. The paired *t*-test was used to compare results within groups and a two-sample analysis of variance to compare results between men and women. All results are expressed as mean ± SD; significance was taken as $P < 0.05$. Spearman's rank correlation (r_s) was used to evaluate the relative change in plasma cholesterol among the subjects for the different dietary procedures.

RESULTS

The changes in body weight and dietary composition of the three groups of subjects are shown in Table 1. The carbo-

TABLE 1

Changes in Dietary Composition over the Five-Week Intervention in Subjects Fed a Very Low Fat Vegetarian Diet and Then Supplemented with Butter, Olive Oil or Safflower Oil in Weeks 4–5[a]

	Total energy (kcal/d)	Carbohy.	Fat	Sat.	Monounsat.	Polyunsat.	Protein	Alcohol	Cholesterol (mg/d)	Fiber (g/d)	Body weight (kg)
		(% total energy)									
Butter group											
Week 1	2117 ± 820	50.6 ± 4.8	32.6 ± 5.9	13.6 ± 2.4	11.3 ± 1.4	7.7 ± 3.7	15.7 ± 3.6	1.1 ± 1.4	212 ± 144	26.5 ± 7.7	67.3 ± 12.1
Week 2	2040 ± 715	75.3 ± 2.7[b]	9.6 ± 2.0[b]	4.1 ±	3.4 ± 0.7[b]	2.2 ± 0.4[b]	13.8 ± 1.3	1.4 ± 1.8	244 ± 29	37.5 ± 14.4[d]	67.2 ± 12.3
Week 3	2066 ± 625	75.7 ± 2.6[b]	9.4 ± 1.7[b]	3.9 ±	3.3 ± 0.5[b]	2.3 ± 0.5[b]	14.1 ± 1.6	0.9 ± 1.3	249 ± 25	37.9 ± 10.3[c]	66.9 ± 11.9[d]
Week 4	2160 ± 775	68.0 ± 3.5[b]	19.1 ± 2.7[b]	10.2 ± 1.6	6.5 ± 0.9[b]	2.3 ± 0.5[b]	13.1 ± 1.9[d]	0.9 ± 1.0	309 ± 39[c]	33.5 ± 8.9[d]	67.2 ± 12.4
Week 5	2215 ± 688	58.7 ± 4.0[c]	28.1 ± 4.0	16.1 ± 2.4	9.6 ± 1.4[d]	2.4 ± 0.6[b]	13.1 ± 1.3[d]	0.6 ± 1.1	371 ± 68[b]	35.1 ± 11.5	66.9 ± 12.0
Olive oil group											
Week 1	2022 ± 608	48.4 ± 8.4	32.6 ± 7.4	15.8 ± 4.6	12.5 ± 3.0	4.4 ± 1.1	16.8 ± 3.0	2.0 ± 1.8	255 ± 163	21.2 ± 7.9	68.0 ± 10.8
Week 2	1975 ± 680	75.2 ± 3.4[b]	8.3 ± 2.2[b]	2.9 ±	2.9 ± 0.9[b]	2.8 ± 0.8[c]	14.1 ± 3.3	2.0 ± 3.5	232 ± 17	38.6 ± 9.6[d]	68.3 ± 10.3
Week 3	1863 ± 500	76.6 ± 3.3[b]	8.2 ± 1.3[b]	2.8 ±	2.8 ± 0.6[b]	2.6 ± 0.3[b]	14.0 ± 2.7	1.4 ± 1.7	230 ± 14	37.0 ± 13.6[d]	67.7 ± 10.1
Week 4	2019 ± 401	66.5 ± 2.7[b]	18.2 ± 1.7[c]	3.8 ±	10.3 ± 1.1	4.1 ± 1.9	12.9 ± 1.6[d]	2.3 ± 2.3	228 ± 20	37.6 ± 8.5[d]	67.7 ± 10.1
Week 5	2195 ± 514	59.7 ± 3.9[d]	27.4 ± 2.5	5.1 ±	17.8 ± 1.7[b]	4.5 ± 0.5	11.8 ± 1.3[c]	1.2 ± 1.9	227 ± 14	35.4 ± 8.8[d]	67.8 ± 9.8
Safflower oil group											
Week 1	2110 ± 524	47.2 ± 7.7	35.5 ± 8.2	15.2 ± 3.9	12.5 ± 3.9	7.7 ± 4.6	15.2 ± 2.3	2.2 ± 3.2	266 ± 142	21.7 ± 7.7	65.6 ± 11.9
Week 2	1741 ± 440[d]	76.5 ± 2.3[b]	8.6 ± 1.5[b]	3.2 ±	3.0 ± 0.4[b]	2.4 ± 0.7[b]	13.2 ± 1.8[d]	1.7 ± 2.4	229 ± 12	32.8 ± 10.7[d]	65.1 ± 11.5
Week 3	1921 ± 690	76.6 ± 3.0[b]	9.2 ± 1.4[b]	3.5 ±	3.2 ± .5[b]	2.5 ± 0.4[b]	13.2 ± 1.8	1.0 ± 1.3	236 ± 18	33.8 ± 11.2	65.2 ± 11.6
Week 4	1995 ± 710	65.6 ± 4.5[b]	19.4 ± 3.0[b]	4.9 ±	5.1 ± 0.7[b]	9.4 ± 2.2	13.3 ± 2.6	1.7 ± 3.8	227 ± 15	33.3 ± 10.1[b]	64.9 ± 11.9[c]
Week 5	2087 ± 484	59.5 ± 3.2	27.2 ± 2.4	5.7 ±	6.6 ± 0.9[b]	14.9 ± 1.8[b]	11.9 ± 1.6[c]	1.5 ± 2.5	225 ± 5	33.3 ± 9.3[c]	65.1 ± 11.8

[a]Mean ± SD, n = 10. Abbreviations: Carbohy. = carbohydrate; Sat. = saturated; monounsaturated = monounsaturated; polyunsat. = polyunsaturated.
[b–d]Significantly different from week 1; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$.

hydrate supplement ensured that the energy intake remained relatively constant throughout the study period despite major changes to the dietary fat composition imposed by the dietary protocol. The mean body weight did not change significantly in the olive oil group, but in both the other groups there was one time point in the 5 wk when there was a significant decrease in body weight for the group (week 3, butter group and week 4, safflower group).

As planned, the proportion of energy derived from fat in weeks two and three fell to just below 10% during the VLF diet, which was compensated for by an increase in carbohydrate using the carbohydrate supplement. The trend toward increased fiber was significant in each of the three groups at most of the time intervals ($P < 0.05$). In the group fed butter, the dietary cholesterol increased in weeks four ($P < 0.05$) and five ($P < 0.001$), and the percentage of fat energy contributed from saturated fatty acids also increased as expected during this time. In the groups fed olive oil or safflower oil, the dietary cholesterol remained constant throughout weeks two to five, inclusive.

The percentage of energy from fat contributed by PUFA, monounsaturated fatty acids and saturated fatty acids changed as expected in weeks four and five on the addition of the different fats. In the butter group, the proportion of saturated fats was higher in week five than during week one (1.2×), while the percentage of energy derived from PUFA was less than 1/3 of the baseline value. Subjects who supplemented their diet with olive oil during weeks four and five of the study had an increased percentage energy from monounsaturated fatty acids in week five compared with the baseline value (1.4×), while there was a 2/3 drop in the proportion of saturated fatty acids in the same period. In the subjects who supplemented their diet with safflower oil, the percentage energy from PUFA in week five was approximately double that of the subjects' usual diet, the proportion of saturated fatty acids dropped by about 2/3 in week five compared with the usual diet and that from monounsaturated fatty acids fell by about one-half in the same period. The mean dietary P/S ratio at baseline was 0.5 for all 30 subjects compared with 0.7 on the VLF diet, and 0.2, 0.9 and 2.6 after 2 wk on butter, olive oil or safflower oil, respectively.

The changes in cholesterol and triglyceride concentrations in lipoprotein lipids over the 5-wk study period are presented in Table 2. Total cholesterol concentrations fell significantly ($P < 0.01$) within one week of commencing the VLF diet by a mean of 0.53 mM for all 30 subjects. After two weeks on the VLF diet, the mean reduction of total plasma cholesterol was 0.60 mM (12%) for all 30 subjects. The decline in total cholesterol was observed in 28/30 subjects, and the range of responses varied from an increase of 0.9 mM to a decline of 1.9 mM (Fig. 1). After two weeks on the VLF diet, LDL-cholesterol in all 30 subjects fell by a mean of 0.38 mM (11%) ($P < 0.01$), while the HDL-cholesterol concentrations also decreased significantly ($P < 0.001$) by a mean of 0.36 mM (26%). These changes were evident after one week on the VLF diet. Plasma triglyceride and VLDL triglyceride both rose significantly ($P < 0.001$) during the 2 wk on the VLF diet in all three study groups. VLDL-cholesterol concentrations

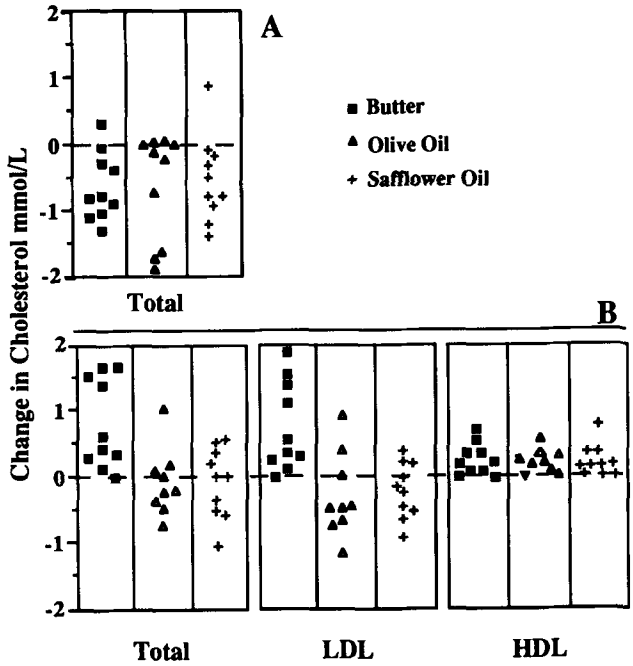

FIG. 1. Changes in plasma total, low density lipoprotein (LDL)- and high density lipoprotein (HDL)-cholesterol levels. A. The difference in total plasma cholesterol between subjects on the usual diet (week 1) and on the very low fat (VLF) diet (week 3) is plotted for each subject. The three columns represent the values for individuals in each of the three diet groups (butter, olive oil and safflower oil). B. The difference in total, LDL- and HDL-cholesterol levels between the VLF diet (week 3) and diets containing butter, olive oil, or safflower oil (week 5) is plotted for each subject. The three columns represent the values for individuals in each of the three diet groups (butter, olive oil and safflower oil).

increased significantly in 2/3 groups on the VLF diet. There was no significant difference between the response of the men and the women to the VLF diet in terms of changes in total plasma cholesterol, LDL-cholesterol or HDL-cholesterol.

In the group supplemented with butter during weeks four and five of the study, the total, LDL- and HDL-cholesterol levels increased significantly each week ($P < 0.01$) compared with the VLF diet (week 3). Total cholesterol had increased by 20% upon the addition of butter for two weeks while LDL- and HDL-cholesterol concentrations increased by 29 and 16%, respectively, during this time. The rise in total and LDL-cholesterol occurred in 9/10 subjects and 7/10 subjects for the HDL-cholesterol (Fig. 1). There was no significant rank correlation for the degree of change in the total plasma cholesterol from the usual diet to the VLF diet compared with the change from the VLF diet to the butter-enriched diet ($r_s = 0.36, P > 0.05$). Values for both total cholesterol and LDL-cholesterol concentrations at the end of week 5 were significantly above the baseline level of this group ($P < 0.05$), which is consistent with the increase in dietary saturated fat and cholesterol in this period. Triglyceride concentrations decreased during weeks 4 and 5 with the addition of butter to the VLF

TABLE 2

Changes in Plasma Lipid Composition over the Five-Week Intervention in Subjects Fed a Very Low Fat Vegetarian Diet in Weeks 2–3 and Then Supplemented with Butter, Olive Oil or Safflower in Weeks 4–5[a]

	Cholesterol				Triglycerides		
	Total (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	VLDL (mmol/L)	Total (mmol/L)	VLDL (mmol/L)	LDL/HDL
Butter group							
Week 1	5.12 ± 0.75	3.47 ± 1.02	1.45 ± 0.50	0.17 ± 0.09	1.00 ± 0.09	0.35 ± 0.13	2.84 ± 1.55
Week 2	4.58 ± 0.50[c]	3.08 ± 0.69	1.19 ± 0.37[d]	0.31 ± 0.17[d]	1.33 ± 0.60[c]	0.58 ± 0.24[d]	3.02 ± 1.59
Week 3	4.48 ± 0.62[c]	3.03 ± 0.83[d]	1.08 ± 0.38[c]	0.38 ± 0.23[d]	1.31 ± 0.45[d]	0.60 ± 0.22[c]	3.27 ± 1.26
Week 4	4.87 ± 0.65[f]	3.39 ± 0.85[g]	1.23 ± 0.30	0.25 ± 0.11[d]	1.25 ± 0.11[d]	0.48 ± 0.18[f,c]	3.05 ± 1.41
Week 5	5.36 ± 1.04[f]	3.91 ± 1.08[f,d]	1.25 ± 0.31[g]	0.42 ± 0.11	1.19 ± 0.41[c]	0.45 ± 0.14[g]	3.52 ± 1.66
Olive oil group							
Week 1	5.26 ± 1.00	3.75 ± 0.76	1.32 ± 0.39	0.19 ± 0.16	1.01 ± 0.35	0.44 ± 0.30	3.02 ± 0.92
Week 2	4.60 ± 1.03[c]	3.31 ± 0.79[d]	1.03 ± 0.37[b]	0.26 ± 0.15	1.20 ± 0.42	0.59 ± 0.31	3.59 ± 1.34
Week 3	4.60 ± 1.08[d]	3.25 ± 0.78	1.02 ± 0.39[c]	0.32 ± 0.30	1.30 ± 0.32[d]	0.63 ± 0.45[d]	3.69 ± 1.77
Week 4	4.73 ± 1.01[d]	3.36 ± 0.78	1.12 ± 0.29[d]	0.26 ± 0.22	0.76 ± 0.18[f]	0.51 ± 0.32[d]	3.17 ± 1.12
Week 5	4.54 ± 0.92[d]	3.02 ± 0.64[c]	1.25 ± 0.37[f]	0.20 ± 0.12	0.85 ± 0.15[f,d]	0.47 ± 0.24	2.62 ± 0.88
Safflower oil group							
Week 1	4.96 ± 0.73	3.29 ± 0.79	1.39 ± 0.33	0.18 ± 0.10	1.03 ± 0.46	0.37 ± 0.21	2.62 ± 1.41
Week 2	4.56 ± 0.89	2.93 ± 0.80[d]	1.07 ± 0.28[b]	0.44 ± 0.51	1.48 ± 0.78	0.73 ± 0.63[d]	2.96 ± 1.38
Week 3	4.45 ± 0.93[d]	2.97 ± 0.83	1.00 ± 0.21[b]	0.40 ± 0.23[c]	1.43 ± 0.57[c]	0.69 ± 0.38[c]	3.14 ± 1.35[c]
Week 4	4.59 ± 0.75	3.07 ± 0.72	1.16 ± 0.32[f,b]	0.39 ± 0.31	1.10 ± 0.44	0.47 ± 0.23	2.89 ± 1.26
Week 5	4.33 ± 0.63[d]	2.83 ± 0.66[c]	1.17 ± 0.34[g,c]	0.28 ± 0.16	1.02 ± 0.44[f]	0.53 ± 0.33[g]	2.74 ± 1.55[g]

[a]Mean ± SD; n = 10. Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein.

[b–d]Significantly different from week 1; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$.

[f,g]Significantly different from week 3; [f]$P < 0.01$; [g]$P < 0.05$.

diet, but they still remained significantly higher than the baseline value ($P < 0.01$).

In contrast to the group supplemented with butter, total cholesterol concentrations remained significantly lower ($P < 0.05$) than the values on the subjects' usual diet when either olive oil or safflower oil were added to the VLF diet (Table 2), although there were different individual responses to the addition of unsaturated oils ranging from a decrease in total cholesterol of 1.1 mM to an increase of 1.1 mM (Fig. 1). For each of the diets containing the added unsaturated oils, 6/10 subjects showed a decrease in the total cholesterol concentration compared with the value on the VLF diet. There was no significant rank correlation for the degree of change in the total plasma cholesterol from the usual diet to the VLF diet compared with the change from the VLF diet to the olive oil-enriched diet ($r_s = -0.04, P > 0.05$). However, there was significant negative correlation in the safflower oil-fed group ($r_s = -0.66$, $P < 0.05$) (*i.e.*, the subjects showing the greatest decrease in plasma cholesterol on the VLF diet showed the greatest rise on the safflower oil diet). The LDL-cholesterol concentrations in week 5 after the addition of either of the unsaturated oils were significantly lower than the values determined in these subjects on their usual diet ($P < 0.01$), although they were not significantly different from the levels on the VLF diet at week 3 of the study. HDL-cholesterol concentrations were significantly higher in week 5 on addition of either olive oil ($P < 0.01$) or safflower oil ($P < 0.05$) compared with the VLF diet at week 3 of the study, by 23 and 17%, respectively.

The LDL/HDL ratios rose following the change from the usual diet to the VLF diet for the 30 subjects (from 2.9 to 3.4, $P < 0.05$). The ratio remained elevated on the butter diet (ratio = 3.5); however, the ratio fell significantly ($P < 0.05$) on the safflower oil diet to a value of 2.7. The ratio fell in 7/9 subjects on the olive oil diet, but the mean value was not significantly different from the value on the VLF diet. Triglyceride concentrations fell during weeks 4 and 5 with the addition of olive oil or safflower oil. For both these groups this decrease was significant when compared with week 3. The mean triglyceride concentration at week 5 in the olive oil group was less than the week 1 baseline values. VLDL triglyceride concentrations followed a similar trend to total plasma triglycerides.

There were significant changes in the fatty acid composition of the plasma PL in the subjects during the VLF period compared with the usual baseline diet (Table 3). The major changes in all three groups of subjects were significant reductions in the percentage of 18:2ω6 ($P < 0.01$) and 20:5ω3 ($P < 0.05$) and significant rises in 16:1 ($P < 0.05$), 20:3ω6 ($P < 0.01$) and 20:4ω6 ($P < 0.05$). There was a decline in the percent of stearic acid which was significant in 2/3 groups ($P < 0.01$). There were rises in palmitic acid (16:0) and docosahexaenoic acid (22:6ω3), which were significant in 2/3 groups ($P < 0.05$). The inclusion of butter in the diet was associated with a normalization of the plasma PL fatty acid profile with the exception of 16:1 and 20:3ω6, which remained significantly elevated ($P < 0.05$). The addition of olive oil led to a significant increase in oleic acid (18:1ω9) ($P < 0.05$) whereas 18:3ω3, 20:5ω3 and docosapentaenoic acids (22:5ω3) remained significantly lower than on the usual diet ($P < 0.05$). The safflower oil-fed group had increased levels of 20:4ω6 ($P < 0.01$) and 22:6ω3 ($P < 0.05$) compared with the usual diet and significantly decreased levels of 16:0, 18:1, 20:5ω3 and 22:5ω3.

There were significant changes in the fatty acid composition of the plasma CE in the subjects during the VLF period compared with the usual baseline diet (Table 4). The major changes in all subjects were reductions in the percentage of 18:2ω6 ($P < 0.01$) and a rise in the percentages of 16:0 ($P < 0.01$), 16:1 ($P < 0.01$), 18:1ω9 ($P < 0.01$), 20:3ω6 ($P < 0.05$) and 20:4ω6 ($P < 0.05$). The proportion of 20:5ω3 decreased significantly in 2/3 groups ($P < 0.05$). The addition of butter had little effect on the fatty acid composition of the CE fraction compared with the composition on the VLF diet, except for a decrease in the proportion of 20:4ω6. The addition of either olive oil or safflower oil led to a normalization of the 16:0 and 16:1 values and a significant reduction in the proportion of 18:3ω3 ($P < 0.05$) and 20:5ω3 ($P < 0.01$) compared with the usual diet. In the case of olive oil, the level of 18:1ω9 ($P < 0.001$) and 20:3ω6 ($P < 0.05$) remained significantly elevated compared with week 1 (baseline), and the proportion of 18:2ω6 rose but was still significantly below the baseline value ($P < 0.001$). In the safflower oil-fed group, the 18:1ω9 and 20:5ω3 proportions fell below the baseline value ($P < 0.001$) and that of 18:2ω6 rose above the baseline ($P < 0.01$). In the latter two groups, the proportion of 20:4ω6 remained significantly elevated above the baseline value ($P < 0.01$).

DISCUSSION

This study was designed to compare the effect of the total fat content (<10% and up to 30% of energy from fat) and the quality of the fat in subjects consuming one egg per day on plasma cholesterol levels. The results indicate that a reduction in the saturated fat content of the diet (<6% dietary energy) either by reducing the total fat content of the diet (VLF diet) or by exchanging saturated fat with unsaturated fat reduced the total plasma cholesterol levels by approximately 12% in normocholesterolemic subjects. The VLF diet reduced the levels of both plasma LDL- and HDL-cholesterol while the 30% fat diets containing monounsaturated fats or PUFA maintained the low LDL-cholesterol levels and increased HDL-cholesterol levels. The 30% fat diet rich in saturated fat (butter) led to an increase in all cholesterol-containing fractions in the plasma.

The design of this study was chosen in order to compare the effect of major changes in both fat content and fat quality over a short time period. Since the usual diet of the subjects contained 34% energy from fat (range 21–47%), it was felt the design would allow, firstly, a comparison between this medium to high-fat diet and the VLF diet, and secondly, that adding different types of fat back to the VLF diet gave an opportunity to examine the effect of *marked* changes in dietary fat quality. For example, the change in saturated fat in the group fed butter was from 14% on the usual diet, to 4% on the VLF diet, then to 16% of energy during the addition of butter to the diet. This design, therefore, allowed a comparison of a substantial decrease

TABLE 3

Fatty Acid Composition of Plasma Phospholipids over the Five-Week Intervention in Subjects Fed a Very Low Fat Vegetarian Diet in Weeks 2–3 and Then Supplemented with Butter, Olive Oil or Safflower Oil in Weeks 4–5[a]

Fatty acid	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+butter)	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+olive oil)	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+safflower oil)
14:0	0.5 ± 0.2	0.4 ± 0.1[d]	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.4	0.4 ± 0.2[g]	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
16:0	26.8 ± 2.0	28.4 ± 2.3	27.6 ± 1.7	26.5 ± 1.5	29.4 ± 1.8[d]	26.9 ± 1.5[g]	27.3 ± 2.0	29.3 ± 2.3[b]	26.2 ± 2.3[c,e]
16:1	1.6 ± 0.3	1.8 ± 0.2[d]	1.8 ± 0.2[d]	1.7 ± 0.4	2.1 ± 0.4[d]	1.4 ± 0.4[e]	1.6 ± 0.4	1.9 ± 0.4[b]	1.4 ± 0.4[f]
18:0	12.0 ± 1.5	11.3 ± 2.1	12.0 ± 1.8[f]	12.7 ± 1.9	11.4 ± 1.8[b]	11.6 ± 2.0	11.3 ± 1.1	10.0 ± 1.3[c]	10.7 ± 1.4
18:1	11.7 ± 1.1	12.3 ± 1.1	12.3 ± 1.3	12.0 ± 1.2	13.1 ± 1.2[d]	14.8 ± 1.9[d]	11.7 ± 1.6	12.2 ± 1.6	10.3 ± 1.7[c,f]
18:2ω6	26.6 ± 3.0	21.2 ± 3.0[c]	23.6 ± 3.8[f]	23.5 ± 1.4	18.1 ± 2.4[b]	21.7 ± 2.5[f]	26.5 ± 2.4	20.9 ± 1.7[b]	29.0 ± 4.4[e]
18:3ω3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1[d]	0.2 ± 0.1[c]	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:3ω6	2.9 ± 1.0	3.9 ± 1.0[c]	3.6 ± 0.9[d]	3.4 ± 0.5	4.4 ± 0.5[c]	3.7 ± 0.6[f]	2.7 ± 0.8	3.5 ± 1.2[c]	2.5 ± 0.9[f]
20:4ω6	10.5 ± 1.9	12.1 ± 2.3[b]	10.7 ± 1.8[f]	11.5 ± 1.9	12.7 ± 1.7[d]	12.5 ± 0.9	10.8 ± 2.2	12.8 ± 2.4[b]	12.2 ± 2.1[c]
20:5ω3	1.0 ± 0.4	0.8 ± 0.3[d]	1.1 ± 0.2[f]	1.5 ± 0.7	0.9 ± 0.4[c]	0.7 ± 0.2[d]	1.1 ± 0.5	0.8 ± 0.3[c]	0.4 ± 0.2[c,f]
22:5ω3	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.1 ± 0.3	0.8 ± 0.2[c,g]	1.0 ± 0.3	1.2 ± 0.3[d]	0.8 ± 0.3[d,f]
22:6ω3	4.2 ± 1.3	4.9 ± 1.6[d]	4.1 ± 1.3[f]	4.3 ± 1.0	4.7 ± 1.0	4.1 ± 0.6	4.3 ± 1.3	5.4 ± 1.3[b]	4.7 ± 1.3[d,f]

[a]Mean ± SD (g/100 g total fatty acids); n = 10/group.

[b–d]Significantly different from week 1; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$.

[e–g]Significantly different from week 3; [e]$P < 0.001$; [f]$P < 0.01$; [g]$P < 0.05$.

TABLE 4

Fatty Acid Composition of Plasma Cholesteryl Esters over the Five-Week Intervention in Subjects Fed a Very Low Fat Vegetarian Diet in Weeks 2–3 and Then Supplemented with Butter, Olive Oil or Safflower Oil in Weeks 4–5[a]

Fatty acid	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+butter)	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+olive oil)	Week 1 (usual diet)	Week 3 (very low fat)	Week 5 (+safflower oil)
16:0	11.0 ± 1.4	13.6 ± 2.6[c]	12.2 ± 1.1[d]	11.6 ± 1.9	14.3 ± 2.2[c]	11.7 ± 0.7[f]	11.2 ± 0.6	12.9 ± 0.8[b]	10.6 ± 0.9[e]
16:1	3.7 ± 0.8	5.6 ± 1.3[b]	5.3 ± 2.3[d]	3.9 ± 0.9	6.6 ± 1.9[c]	3.9 ± 0.9[f]	4.2 ± 1.5	6.0 ± 2.0[b]	3.3 ± 1.1[d,e]
18:1	18.1 ± 2.7	21.7 ± 1.9[c]	20.7 ± 2.7	20.2 ± 1.4	24.3 ± 2.8[c]	25.8 ± 1.7[b]	18.6 ± 2.6	21.7 ± 2.3[c]	14.6 ± 2.6[b,e]
18:2ω6	56.5 ± 4.8	46.0 ± 5.1[b]	49.4 ± 6.2[d,g]	52.4 ± 3.1	41.4 ± 4.3[b]	46.6 ± 2.9[b,f]	55.2 ± 4.2	45.4 ± 4.8[b]	60.2 ± 5.0[c,e]
18:3ω3	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1[g]	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1[d]	0.5 ± 0.2	0.6 ± 0.4	0.3 ± 0.1[c]
20:3ω6	0.7 ± 0.2	0.9 ± 0.4[d]	0.8 ± 0.3[g]	0.7 ± 0.2	1.0 ± 0.2[c]	0.9 ± 0.1[c,f]	0.6 ± 0.2	0.9 ± 0.2[b]	0.6 ± 0.2[f]
20:4ω6	6.4 ± 1.3	8.4 ± 2.3[c]	7.2 ± 1.7[g]	6.7 ± 1.9	8.0 ± 2.2[d]	8.0 ± 1.7[c]	6.6 ± 1.3	8.9 ± 2.4[b]	7.9 ± 1.9[c]
20:5ω3	0.7 ± 0.3	0.6 ± 0.1	0.9 ± 0.2[e]	0.9 ± 0.4	0.6 ± 0.2[d]	0.5 ± 0.2[c]	0.8 ± 0.3	0.6 ± 0.3[c]	0.3 ± 0.2[c,f]
22:6ω3	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	1.0 ± 0.6	0.7 ± 0.2

[a]Mean ± SD (g/100 g total fatty acids); n = 10/group.

[b–d]Significantly different from week 1; [b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.05$.

[e–g]Significantly different from week 3; [e]$P < 0.001$; [f]$P < 0.01$; [g]$P < 0.05$.

and then an increase in saturated fat. Other designs, which start from modest to high fat levels, do not allow comparisons of changes in saturated fat of this magnitude. If the butter diet had merely followed the subjects' usual diet, without the intervention of the VLF diet period, the change in saturated fat would only have been from 14% (usual diet) to 16% (butter diet). Because of the variations in the fat intake of the subjects on their usual diet, such a change would have meant a reduction in saturated fat for some subjects and an increase in saturated fat for others, thus leading to potentially different effects on plasma cholesterol levels. Although the dietary periods in the study were only of 1–2 wk duration, others have reported that serum cholesterol levels can reach a steady state within 7–14 d of commencing a new dietary regime (4–6) and maintain these levels over several months (6,9). In confirmation of these observations, the changes in plasma cholesterol in the present study took place rapidly with the greatest changes occurring in the first 7 d on the VLF diet. In contrast, addition of butter at 10% energy for 7 d and then at 20% energy for an additional 7 d was associated with a continuous increase in the plasma cholesterol concentration. We acknowledge that the dietary periods were of a relatively short duration, and we cannot exclude the possibility that the cholesterol levels measured were not in a steady state. The study design chosen involved substantial changes to the level and quality of fat, and in such circumstances we were concerned about poor compliance to these diets if the diet periods had been extended in time.

When subjects were on the VLF diet, there were reductions in both the LDL- and HDL-cholesterol levels as reported by our group and others in previous studies with diets very low in fat (5,8,10,11). It would be expected that both the decrease in saturated fat from 15 to 3% and the increase in fiber from 23 to 35 g/d (12,13) would have contributed to this plasma cholesterol reduction. VLF diets (high-carbohydrate diets) are frequently associated with increased VLDL production and increased plasma triglyceride concentrations (14), as was observed in the groups on the VLF diet. The carbohydrate increased from 48 to 76% of energy in this period. The reduced HDL-cholesterol concentration we observed may have been due, in part, to an exchange of triglyceride and CE between VLDL and HDL, resulting in a relative depletion of cholesterol from the HDL particle (15). Recent work investigating the turnover of apolipoprotein A (transport and catabolic rates), in a group of subjects who consumed both low- and high-fat diets, has suggested that mechanisms which result in the decrease in HDL-cholesterol levels observed in the subjects on the VLF diet differ from those mechanisms which lead to low HDL-cholesterol levels in some individuals on high-fat diets (10). There has been some discussion recently about the HDL-cholesterol lowering effect of low-fat diets with Sacks and Willett (3) arguing that, on balance, there is insufficient evidence to conclude that lowering of HDL-cholesterol by reduction in the total fat intake is innocuous. However, populations which habitually consume low-fat diets have low levels of both LDL- and HDL-cholesterol and also have low rates of coronary heart disease (16).

The effect of increasing the fat content of the diet from below 10 to 30% over the final 2 wk of the study depended on the quality of the fat introduced. The inclusion of butter, which increased the saturated fat and the cholesterol content of the diet, was associated with significant rises in total, LDL- and HDL-cholesterol levels; both the total and LDL values significantly exceeded the prediet values. In contrast, the unsaturated oils did not significantly change the total cholesterol concentration, but there were significant increases in the HDL-cholesterol fraction on both diets ($P < 0.05$), which were partially compensated for by nonsignificant decreases in the LDL-cholesterol values. The main difference between the olive oil and safflower oil diets was that the HDL-cholesterol was not significantly different from pretrial values in the olive oil group, whereas the HDL-cholesterol was still 16% lower than the starting values ($P < 0.01$) in the safflower group. Although it is recognized that dietary linoleic acid at 30–50% energy lowers both LDL- and HDL-cholesterol (17,18), there is evidence that at more realistic intakes (*e.g.*, 10% energy) there are effects only on the LDL-cholesterol concentrations (19,20). However, a more recent long-term study of a comparison between olive oil and sunflower oil, in which the PUFA intake was 12–13% energy, showed that there were significantly higher concentrations of HDL-cholesterol in the subjects on the olive oil diet (21). The present result, at 20% energy safflower oil, indicates a difference between the olive oil and safflower oil groups, but whether this is the result of the high level of linoleic acid, the relatively short term nature of this experiment or the effect of individual responses is not clear.

These results highlight the observations of others who have reported a wide variability in the response of plasma cholesterol concentration to changes in diet between individuals (22–24). Apolipoprotein polymorphism, particularly apoE and apoB phenotypes, may contribute to the variation in responses of individuals to changes in the amounts of dietary fat and cholesterol (25,26). Apoprotein phenotypes were not measured in the present study, and therefore, it is not possible to make generalizations about the subjects' individual variations.

Subjects experienced difficulty in maintaining their weight on the VLF diet, and this result is similar to that of other investigators who have reported low-fat diets to be an effective means of achieving weight reduction even when no limitations are placed on the quantity consumed (27). However, a diet which replaces saturated fat with monounsaturated fats or PUFA rather than carbohydrate is easier to comply with and, therefore, presents a more realistic and achievable goal for public health messages. A moderate-fat diet allows greater scope for variety than a VLF diet, thereby helping to ensure an adequate supply of nutrients. Such a diet may also minimize both the increase in triglycerides and the decrease in HDL-cholesterol accompanying substantial increases in carbohydrate intake.

There were changes in the fatty acid composition of the plasma CE and PL in subjects on the VLF diet which were similar to those reported in our previous studies on VLF diets (4,5,8). These included increases in 16:0 and 16:1 and decreases in the proportion of 18:2ω6. The former in-

creases are presumably the result of increased synthesis of fat from the high carbohydrate diet and the latter decrease maybe due to the very low level of the essential 18:2ω6 in the diet. Additionally, the VLF diet was associated with increases in 20:3ω6, 20:4ω6 and decreases in 20:5ω3 in both lipid fractions and increases in 22:6ω3 in the PL. These latter changes may be due to the fact that all subjects were consuming one egg/day which provided 20:3ω6, 20:4ω6 and 22:6ω3 at levels of 8, 77 and 31 mg per 50 g egg, respectively (7). We have previously reported that VLF vegetarian diets (egg-free) consumed for a similar 2-wk period were associated with decreases in 18:2ω6 and 20:5ω3 only, whereas VLF diets containing low levels of long-chain PUFA, such as 20:4ω6, 20:5ω3 and 22:6ω3 (20–80 mg long-chain PUFA/100 g edible food) derived from either lean meat or fish, were associated with increases in the plasma PL and CE levels of 20:3ω6, 20:4ω6 and 22:6ω3 (8).

The addition of the three dietary fats/oils to the VLF diet resulted in interesting changes in the plasma lipid fatty acid profiles, particularly for the 20 carbon PUFA. The butter diet resulted in a raised 20:3ω6 and 20:5ω3 and decreased 20:4ω6 compared with the VLF diet. In contrast, both the olive and safflower oil diets decreased 20:3ω6 and 20:5ω3 levels and maintained the elevated 20:4ω6 level seen on the VLF diet. The latter two diets also decreased the level of 18:3ω3. Lasserre *et al.* (28) have previously reported similar effects of diet on the 20 carbon PUFA of plasma PL in subjects fed diets rich in milk fat or sunflower oil, respectively, for 5 mon. A possible explanation for these results is based on the known competition between oleic, linoleic and linolenic acids for metabolism *via* the Δ6 desaturase (29). Diets rich in milk fat contain a low level of linoleic acid and a low 18:2ω6:18:3ω3 ratio (about 3:1) which would enhance the formation of 20:5ω3 relative to 20:4ω6 (30). In contrast, the diets rich in olive oil and safflower oil were essentially devoid of 18:3ω3, and thus the high level of 18:1ω9 or the 18:2ω6 would effectively block metabolism of any small quantity of linolenic acid to 20:5ω3 (31).

There is general agreement that the ratio of the ω6 to ω3 PUFA is too high in the current Western diet. It has been postulated that this dietary imbalance leads to a tissue PUFA imbalance and a consequent exaggerated metabolism of 20:4ω6 to eicosanoids (32). On this basis there is concern that diets which raise tissue 20:4ω6 relative to 20:5ω3 might be pro-thrombotic since increased platelet 20:4ω6 could lead to an increased production of thromboxane A_2 with resultant platelet aggregation (33). On the other hand, 20:5ω3 has been shown to effectively reduce production of thromboxane by platelets and to reduce platelet aggregation (34). The present results demonstrate that the VLF diet and the diets containing olive oil or safflower oil increased the 20:4ω6/20:5ω3 ratio in the plasma PL. In addition to these two 20 carbon PUFA, it may also be necessary to consider the plasma levels of 20:3ω6 since this fatty acid is a precursor of prostaglandin E1, which prevents platelet aggregation and thus might be potentially antithrombotic (35). The VLF and the butter diets increased the 20:3ω6 level in the plasma lipids, whereas both oils reduced the level of this PUFA. Although the changes described in the proportion of plasma PL PUFA in this study were quite small, we have preliminary evidence that diets which induce similarly minor changes in C20 PUFA are associated with significant changes in the *in vivo* production of prostacyclin (as measured by gas chromatography/mass spectrometry of the main urinary metabolite; Mann, N.J., and Sinclair, A.J., unpublished data). These data suggest that advice on plasma lipid lowering should also take into account the effect of the diet on the fatty acid profile of the plasma lipids. To establish the biological significance of the present dietary changes in plasma PUFA, it will be necessary to determine the effects on eicosanoid production and platelet aggregation.

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Interactions Between Medium-Chain and Long-Chain Triacylglycerols in Lipid and Energy Metabolism in Growing Chicks

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The combined effects of dietary medium-chain triacylglycerols (MCT) and long-chain triacylglycerols (LCT) on lipid and energy utilization in chicks were investigated. Corn oil was used as the LCT source, and trioctanoin (8:0) was used as the MCT source. The efficiency of dietary energy utilization (such as metabolizable energy values and fat and energy retention) decreased linearly as the level of MCT increased, but the efficiency of dietary protein utilization (protein retained per protein consumed) was not affected in a consistent manner. Fecal saturated and monounsaturated fatty acid composition was dependent on the dietary fatty acid composition, whereas fecal linoleic acid content was not. It is concluded that dietary MCT and LCT influence each other to some degree, with respect to protein and lipid metabolism in chicks. Moreover, in most cases, the nutritional characteristics of each triacylglycerol, including food efficiency and fat and energy retention, are independent of each other in growing chicks.

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In chicks, a high level of long-chain triacylglycerols (LCT) in the diet was shown to increase the efficiency of energy utilization, but the LCT diet has a tendency to cause fat deposition in the body compared to medium-chain triacylglycerols (MCT) diet. However, the efficiency of protein utilization is improved by dietary MCT (1). Metabolizable energy (ME) (1) and net energy (NE) (2) values for MCT are lower than those for LCT. The metabolism of MCT is carnitine-independent and does not require chylomicron formation. MCT are easily oxidized and utilized as fuel, and have little tendency to deposit as fat (3). On the other hand, dietary LCT alleviate essential fatty acid deficiency (4). High-fat diets increase peroxisomal ß-oxidation activity in rats (5), but the contribution of peroxisomal ß-oxidation to total hepatocellular long-chain fatty acid (LCFA) oxidation depends on both the concentration and the chain length of the fatty acids involved (for a review, see Ref. 6). Thus, dietary MCT have both advantages and disadvantages as a dietary fat source. In order to take full advantage of the nutritional characteristics of both MCT and LCT, further insights must be gained.

It has been demonstrated that dietary MCT and LCT mutually affect each other. For instance, when MCT were administered together with LCT, oxidation was slowed down (7), and release of medium-chain fatty acids (MCFA) into the bloodstream was delayed (8). When LCT and MCT were ingested simultaneously, the energy absorbed was greater than that when either one of the fats was ingested alone (9).

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Abbreviations: CCK: cholecystokinin; LCFA, long-chain fatty acid(s); LCT, long-chain triacylglycerol(s); MCFA, medium-chain fatty acid(s); MCT, medium-chain triacylglycerol(s); ME, metabolizable energy; NE, net energy.

In the present study we investigated the effects of a diet containing MCT and LCT on the lipid and energy metabolism in chicks.

MATERIALS AND METHODS

One-day-old Single Comb White Leghorn male chicks were given a commercial starter diet for seven days. The diet consisted of 21.5% crude protein and 12.1 kJ/g ME (Marubeni Shiryo Ltd., Tokyo, Japan). On day 8, the chicks were individually weighed after fasting overnight and were divided into six groups of five chicks each, so that mean body weights per group were as uniform as possible. The chicks in the sixth groups were killed by cervical dislocation and were used to determine base values. The remaining five groups of chicks were individually housed in stainless-steel metabolic cages and were fed *ad libitum* the experimental diets from day 8 to day 18.

Table 1 shows the composition of the experimental diets. Corn oil was used as the LCT source and Coconado RK® (glyceryl tricaprylate, 8:0; donated by Kao Corporation, Wakayama, Japan) was used as the MCT source. The dietary oil (200 g/kg diet) contained MCT/LCT combinations in five different proportions, namely 0:200, 50:150, 100:100, 150:50 or 200:0 g MCT/LCT/kg diet. The 200 g MCT/kg diet thus was devoid of linoleic acid.

The chicks were killed by decapitation on day 18. The carcass was minced in a meat grinder, the mince frozen in liquid nitrogen, minced for the second time and then dried at 55°C for 48 h. Nitrogen (N) in the diets and carcasses was determined by the Kjeldahl procedure, and protein was calculated as N × 6.25. 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 the values for the initial body composition from the final experimental values. The energy content of the chicks was calculated using the values 39.12 and 23.68 kJ/g for fat and protein, respectively (10). Droppings were collected during the last three days to determine the N-corrected ME values of the diets (11). The trays in which the droppings were collected were filled with 0.05% HCl solution to prevent N loss and fecal fat saponification. 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-Moller *et al.* (12) and measured enzymatically by a Total Bile Acid Test® (Wako Pure Chemicals, Osaka, Japan). Fecal fat was extracted as was fat from carcasses. The total lipid extract was filtered, dried under a gentle stream of nitrogen and the residual lipids were redis-

TABLE 1

Composition of Experimental Diets (g/kg)

	MCT/LCT ratio[a]				
Ingredient	0:200	50:150	100:100	150:50	200:0
Isolated soybean protein			226		
Mineral mixture[b]			58.8		
Vitamin mixture[c]			2		
Choline chloride			1.5		
Inositol			1		
L-Methionine			2.9		
L-Threonine			1.2		
Glycine			4.2		
Cellulose			100		
Corn starch			402.4		
Corn oil	200	150	100	50	0
RK®[d]	0	50	100	150	200
Dietary fatty acid composition (%)					
8:0	0	25.0	50.0	75.0	100
16:0	8.19	6.14	4.10	2.05	0
16:1n-7	0.070	0.053	0.035	0.018	0
18:0	3.72	2.79	1.86	0.930	0
18:1n-9	27.30	20.48	13.65	6.82	0
18:2n-6	58.03	43.52	29.02	14.51	0
18:3n-3	1.06	0.80	0.53	0.265	0
20:3n-9	1.37	1.03	0.685	0.343	0
20:4n-6	0.252	0.19	0.126	0.063	0

[a]MCT, medium-chain triacylglycerols; LCT, long-chain triacylglycerols.

[b]Contained 20.7 g $CaHPO_4 \cdot 2H_2O$, 14.8 g $CaCO_3$, 10 g K_2HPO_4, 3 g KCl, 6 g NaCl, 3 g $MgSO_4$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.35 g $MnSO_4 \cdot 5H_2O$, 2.6 mg KI, 40 mg $CuSO_4 \cdot 5H_2O$, 62 mg ZnO, 1.7 mg $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg Na_2SeO_3, 0.93 mg $CoCl_2$.

[c]Contained 15 mg calcium pantothenate, 6 mg riboflavin, 4 mg pyridoxine hydrochloride, 40 mg nicotinic acid, 1.5 mg folic acid, 0.2 mg biotin, 0.02 mg cyanocobalamin, 3 mg thiamin hydrochloride, 200 ICU vitamin D_3, 0.5 mg vitamin K_3, and 1.93 g glucose. The DL-α-tocopheryl acetate (10 IU) and retinyl acetate (1700 IU) were dissolved in glyceryl tricaprylate or corn oil.

[d]Coconado RK®, glyceryl tricaprylate (Kao Corp., Wakayama, Japan).

solved in 2.8% sodium methoxide to obtain fatty acid methyl esters. The solution was incubated at 45°C for 2 h and extracted three times with 2 mL of hexane. An aliquot of the hexane solution was used for determining the fatty acid composition. 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. The fatty acid composition of the diet was analyzed in the same manner as described for feces.

The data were subjected to analysis of variance, and the significance of differences between means was determined by Duncan's multiple range test using a commercially available statistical analysis package (SAS, 1985; Ref. 13). Regression equations were also fitted to the data.

RESULTS

The effect of dietary MCT and LCT at different ratios on body weight gain, food intake, food efficiency, protein retention and protein utilization is shown in Table 2. All parameters, except for the efficiency of protein utilization, decreased linearly as the proportion of MCT in the diet increased. The efficiency of protein utilization was somewhat improved at the 50:150 and 150:50 ratios, compared with LCT and MCT alone.

Table 3 gives the energy utilization values of diets containing LCT and MCT in various proportions. ME values and ME intake, fat and energy retention, and efficiency of energy utilization decreased linearly as the proportion of dietary MCT increased.

Table 4 shows total fecal fat content and bile acid secretion for chicks given diets with different MCT/LCT ratios. No significant differences were observed between the different ratios in both parameters.

The effects on fecal fatty acid composition are shown in Table 5. The levels of fatty acids with 8:0, 10:0, 12:0 carbon chains increased linearly as MCT levels increased. The levels of 16:0, 18:1n-9, 20:3n-9 and 20:4n-6 decreased linearly as the MCT levels increased. The excretion of linoleic acid (18:2n-6) was not changed as the ratio of MCT/LCT was altered.

TABLE 2

Body Weight Gain, Food Intake, Food Efficiency, Protein Retention and Efficiency of Protein Utilization of Chicks Fed Diets Containing Different Ratios of Medium- (MCT) and Long-Chain Triacylglycerol (LCT)[a]

Parameter	MCT/LCT ratio (g/kg diet) 0:200	50:150	100:100	150:50	200:0	Pooled SEM	Regression equation	SE of intercept	SE of slope	R^2 value	P value
Body weight gain (g/d)	8.96^b	8.48^b	7.02^c	7.12^c	5.70^d	0.41	y = 9.03 – 0.0158x	0.316	0.0026	0.618	<0.001
Food intake (g/d)	14.8^b	$13.9^{b,c}$	$12.4^{c,d}$	$12.6^{c,d}$	11.4^d	0.51	y = 14.6 – 0.0162x	0.385	0.0031	0.535	<0.001
Food efficiency	0.606^b	0.607^b	0.565^b	0.563^b	0.500^c	0.01	y = 0.619 – 0.0005x	0.012	0.0001	0.549	<0.001
Protein retention (g/d)	1.54^b	1.54^b	$1.33^{c,d}$	$1.41^{b,c}$	1.18^d	0.06	y = 1.57 – 0.0017x	0.050	0.0004	0.436	<0.001
Efficiency of protein utilization[e]	$0.541^{b,c}$	0.590^d	$0.553^{b,c,d}$	$0.579^{c,d}$	0.533^b	0.11					

[a]Values are means of five birds.

[b,c,d]Values having different superscript letters are significantly different at $P < 0.05$. Details of triacylglycerol sources are described in Table 1.

[e]Efficiency of protein utilization equals protein retention/protein intake.

TABLE 3

Fat and Energy Retention, Metabolizable Energy (ME) Value and ME Intake of Chicks Fed Diets Containing Different Ratios of Medium- (MCT) and Long-Chain Triacylglycerol (LCT)[a]

Parameter	MCT/LCT ratio (g/kg diet) 0:200	50:150	100:100	150:50	200:0	Pooled SEM	Regression equation	SE of intercept	SE of slope	R^2 value	P value
ME value (kJ/g)	15.9^b	16.0^b	15.2^c	15.2^c	14.5^d	0.15	y = 16.1 – 0.0069x	0.132	0.0011	0.645	<0.001
ME intake (kJ/d)	234^b	223^b	$189^{c,d}$	192^c	165^d	8.06	y = 234 – 0.338x	6.35	0.0518	0.648	<0.001
Fat retention (g/d)	1.56^b	1.34^b	0.81^c	0.78^c	0.38^d	0.11	y = 1.56 – 0.0059x	0.085	0.0007	0.758	<0.001
Energy retention (kJ/d)	97.7^b	88.9^b	63.2^c	63.8^c	42.7^d	5.29	y = 98.3 – 0.270x	4.21	0.0344	0.728	<0.001
Efficiency of energy utilization[e]	0.415^b	0.399^b	0.333^c	0.330^c	0.258^d	0.01	y = 0.424 – 0.0008x	0.0120	0.0001	0.728	<0.001

[a]Values are means of five birds.

[b,c,d]Values having different superscript letters are significantly different at $P < 0.05$. Details of triacylglycerol sources are described in Table 1.

[e]Efficiency of energy utilization equals energy retained/ME intake.

TABLE 4

Total Fecal Fat and Fecal Bile Acids of Chicks Fed Diets Containing Medium- (MCT) and Long-Chain Triacylglycerol (LCT) at Different Ratios[a]

Parameter	MCT/LCT ratio (g/kg diet)					Pooled SEM
	0:200	50:150	100:100	150:50	200:0	
Fecal fat (%)	6.48	5.90	6.82	5.94	6.30	0.482
(mg fat)	27.58	24.31	27.95	24.15	23.40	0.091
(mg/g fat intake)	9.80	8.40	11.2	9.2	9.6	0.008
Bile acid (%)	0.138	0.084	0.079	0.106	0.094	0.03
(mg/d)	5.98	3.29	3.22	4.19	3.37	1.08
(mg/g fat intake)	2.05	1.22	1.26	1.53	1.41	0.412

[a]Values are mean of five birds. Details of triacylglycerol sources are described in Table 1.

The levels of total saturated fatty acids (8:0 + 12:0 + 14:0 + 16:0 + 18:0) increased linearly while the levels of total monounsaturated fatty acids (16:1n-7 + 18:1n-9) decreased linearly as the MCT levels increased. However, the levels of the polyunsaturated fatty acids (n-6; 18:2n-6 + 18:3n-6 + 20:4n-6) were not altered by a change in MCT/LCT ratio.

DISCUSSION

In the diet containing MCT alone as fat source, the requirement for linoleic acid (10 g/kg diet; National Research Council; Ref. 14) was not met. However, symptoms of linoleic acid deficiency, such as growth depression, did not become apparent within the short experimental period. Therefore, the effects seen in the present study were attributed to the nutritional characteristics of MCT *per se*. Feeding the MCT diet reduced the food intake of chicks when compared to those in the LCT diet (1). In the present study an increase in the levels of MCT linearly decreased the food intake by the chicks (Table 2); thus, most of the parameters measured were affected by food intake. The depressive effect of dietary MCT on food intake by chicks has been attributed to the enhanced release of cholecystokinin (CCK); a known satiety hormone (15). Furuse *et al.* (16) have shown, however, that injection of Devazepide, a type A receptor antagonist of CCK, did not block the decrease in food intake caused by MCT in chicks. Denbow *et al.* (17) have shown that intrahepatic administration of MCT suppressed food intake of birds within 1 h, whereas intragastric infusion of MCT resulted in delayed reduction in food intake. The rate of absorption of MCT from the gut could be one of the satiety factors (17). Another reason for the reduced food intake by chicks could be the unpalatability of the MCT diet; chicks preferred the LCT diet when allowed to choose between the MCT and LCT diets (16). This, however, was not true for rats. Given a choice, rats did not show a preference for the LCT diet over the MCT diet (18).

The ME values of the diets also decreased as the dietary MCT concentration increased. According to Clark and Holt (9), the energy absorbed was greater when a combination of MCT and LCT was used than when either fat was ingested alone. Furuse *et al.* (1) calculated the ME of MCT to be 30.7 kJ/g (0.82 of that of LCT, 37.5 kJ/g; Ref. 19). In the present study, no improvement of energy utilization was seen with any combination of MCT and LCT, because as the levels of MCT increased, the ME of the diet decreased. Thus, energy utilization in chicks might be dependent on the energy value of each fat source. As a result, the ME intake was synergistically decreased by both the reduction in food intake and in ME values. All parameters except for the efficiency of protein utilization were associated with the ME intake (equations not shown). It was also reported, however, that when chicks were given the same amount of food (similar ME intake between MCT and LCT groups), fat retention remained low with the MCT diet (20). MCT are hydrolyzed by intestinal enzymes and pancreatic lipase more rapidly than LCT (21), and MCFA are oxidized in larger amounts and more rapidly than are LCFA; thus energy expenditure from MCT is greater (22). In fact, not only the ME value, but also the ME value of MCT is lower than that of LCT in growing chicks (2).

Previous studies have shown that MCT improved the efficiency of protein utilization in chicks (1,20). In these two previous experiments, the MCT diet also contained LCT (180 g MCT and 20 g LCT/kg diet). In the present study, no significant difference was seen in the efficiency of protein utilization between the diets containing LCT alone and MCT alone. However, some combinations of LCT and MCT improved the efficiency of protein utilization, the reason for which is presently not understood. In humans, it was shown that the nitrogen balance was better when total parenteral nutrition patients received lipids containing MCT rather than when LCT alone was given (23).

In the present study, the levels of fat and bile acids in the droppings were measured to evaluate the combined effect of MCT and LCT on the excretion of fatty acids and bile acids that may have resulted from metabolic changes in the chicks. However, there were no differences in the bile acid excretion at different combinations of diet on MCT and LCT. Rubin *et al.* (24) demonstrated that infusion of LCT or MCT, or of a mixture of MCT and LCT, as emulsions did not affect bile flow and bile acid secretion in rats. In chickens, bile flow in the cystic duct was not affected by the dietary fat sources (Mabayo, Furuse and Okumura, unpublished data), although MCT were potent stimulators of CCK secretion (15). LCT require bile acids for micelle formation to facilitate its absorption, whereas MCT do not (25). It was concluded, however, that the reabsorption of bile acids might be independent of the dietary fat source. There were no differences in the amount of fat excreted between the different treatments, which may imply that in chicks, total absorption of dietary fat was probably the same. Possible differences between the MCT and LCT could be explained by different rates of absorption from the gut, by different hepatic fatty acid synthesis and fatty acid oxidation, and by differences in the fate of the fatty acids released from triacylglycerols in the peripheral tissues.

TABLE 5

Fatty Acid Composition (%) of Fecal Fat of Chicks Fed Diets Containing Medium- (MCT) and Long-Chain Triacylglycerol (LCT) at Different Ratios[a]

Parameter	MCT/LCT ratio (g/kg diet) 0:200	50:150	100:100	150:50	200:0	Pooled SEM	Regression equation	SE of intercept	SE of slope	R^2 value	*P* value
8:0	1.03[e]	14.94[d,e]	26.47[c,d]	41.33[c]	69.76[b]	5.62	y = –2.06 + 3.28x	4.37	0.3566	0.786	<0.001
10:0	0.284[c]	0.555[c]	0.871[c]	1.05[c]	3.21[b]	0.549	y = –0.074 + 0.127x	0.440	0.0359	0.351	<0.01
12:0	0.151[c]	0.168[c]	0.227[b,c]	0.186[c]	0.541[b]	0.107	y = 0.095 + 0.016x	0.084	0.0069	0.190	<0.05
14:0	0.448	0.403	0.485	0.284	0.762	0.192					
16:0	19.40[b]	13.01[c]	9.79[c,d]	6.19[d]	5.76[d]	2.10	y = 17.6 – 0.682x	1.60	0.1306	0.543	<0.001
16:1n-7	0.540[b,c]	0.173[c]	0.478[b,c]	0.115[c]	0.725[b]	0.142					
18:0	11.02	7.73	6.46	4.17	6.76	1.55					
18:1n-9	38.51[b]	29.63[c]	22.19[d]	15.88[d]	5.25[e]	2.16	y = 38.3 – 1.61x	1.60	0.1303	0.868	<0.001
18:2n-6	22.18	28.90	30.12	28.97	4.93	6.86					
18:3n-6	0.242	0.204	0.176	0.132	0.308	0.078					
18:3n-3	2.05	1.64	1.31	0.741	1.33	0.399					
20:3n-9	1.57[b]	1.40[b]	0.815[c]	0.727[c]	0.273[c]	0.181	y = 1.61 – 0.065x	0.135	0.0111	0.604	<0.001
20:4n-6	2.58[b]	1.18[b,c]	0.612[c]	0.234[c]	0.404[c]	0.497	y = 2.06 – 0.106x	0.388	0.0317	0.328	<0.001
SFA[g]	32.33[d]	36.82[c,d]	44.30[c,d]	53.21[c]	86.78[b]	5.35	y = 25.6 + 2.51x	4.80	0.3918	0.640	<0.001
MUFA[h]	39.05[b]	29.81[c]	22.67[d]	15.99[e]	5.98[f]	2.16	y = 38.7 – 1.60x	1.58	0.1292	0.869	<0.001
PUFA (n-6)[i]	25.00	30.33	30.91	29.34	5.64	6.45					

[a]Values are means of five birds.

[b–f]Values having different superscript letters are significantly different at $P < 0.05$. Details of triacylglycerol sources are described in Table 1.

[g]Saturated fatty acid (SFA) = 8:0 + 12:0 +14:0 + 16:0 + 18:0.

[h]Monounsaturated fatty acid (MUFA) = 16:1n-7 + 18:1n-9.

[i]Polyunsaturated fatty acid (PUFA) (n-6) = 18:2n-6 + 18:3n-6 + 20:4n-6.

These factors have been pinpointed in chicks fed dietary MCT when food intake and fat retention in the body were decreased, but, conversely, liver fat was increased (1) when compared to LCT. Fat in the feces is present because of incomplete absorption of dietary fat, and partly because of intestinal lipid secretions and the presence of desquamated cells derived from endogenous sources. No specific effect was seen at any combination of the two dietary triacylglycerols on the content of fecal fat; however, there was an effect on specific fatty acids. Octanoic acid (8:0) is absorbed efficiently and rapidly, and it probably does not accumulate in the intestinal mucosa (26); hence, the fecal 8:0 seen in the present study may have been due to the high level of fat present in the diet. The increasing levels of 10:0 and 12:0 seen as dietary MCT levels were increased may have been due to elongation of 8:0 prior to completion of ß-oxidation and may have been the result of intestinal secretion. In the dietary fat, 16:0, 20:3n-9 and 20:4n-6 were present at decreasing levels as the MCT level increased. A similar trend was seen in the fecal fatty acid composition. Linoleic acid (more than 50% of the fatty acid content of corn oil used as LCT source) was not excreted at variable levels into the feces, whereas 18:1n-9 was excreted in a dose-dependent manner. This could mean that 18:2n-6 is absorbed better than 18:1n-9. The predominant mechanism of linoleic acid absorption at high concentrations is thought to be simple diffusion (27), resulting in a linear relationship between linoleic acid concentrations and transport rates (28). Oleic acid was well utilized by chicks with an absorbability of 88% (29). However, the uptake of oleic acid by jejunal mucosal cells is mediated by a membrane-bound fatty acid binding protein, and addition of linoleic acid decreased oleic acid absorption rates (30) accounting for the differences in excretion between oleic acid and linoleic acid. In the present study, the excretion of 8:0 in high concentrations relative to fecal fat raises questions in regard to the absorption mechanism of this fatty acid. Although the absorption of 8:0 was the same after total bile diversion as it was before (31), it is possible that micelle formation is required when 8:0 is given in large amounts. Bloom *et al.* (32) reported that MCT were more completely hydrolyzed than LCT and that their fatty acids were rapidly absorbed *via* the portal vein. Intraduodenal infusion of trioctanoin reduced the steady state absorption of oleic acid, but the absorption of 8:0 was not inhibited by oleic acid. This may imply a competition for some membrane site which may be involved in the mucosal uptake of these fatty acids (9). There might be other mechanisms involved in the absorption of 8:0 when given in high concentration, although Stremmel (30) suggested that 8:0 does not interfere with micelle formation and is taken up by a transport mechanism other than fatty acid binding membrane protein.

We conclude that dietary MCT and LCT affect each other with respect to protein and lipid metabolism in chicks. However, in most cases, the nutritional characteristics of each triacylglycerol, including food efficiency and fat and energy retention are independent of each other.

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COMMUNICATION

The Acute Effects of a Single Very High Dose of n-3 Fatty Acids on Plasma Lipids and Lipoproteins in Healthy Subjects[1]

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Forty healthy volunteers were allocated in a double blind, randomized study to receive either 20 g of n-3 polyunsaturated fatty acids (PUFA) or 20 g of n-6 PUFA at their evening meal. The effect on plasma lipids and lipoproteins of this single dose of fish oil *vs.* corn oil was studied the next morning, 14 h after ingestion. Plasma triglycerides and very low density lipoprotein-cholesterol significantly decreased (33%) after n-3 PUFA ($P < 0.001$), and significantly ($P < 0.01$) more than after intake of n-6 PUFA. The decrease in plasma triglycerides after n-3 PUFA ingestion was more pronounced in subjects with higher baseline levels of triglycerides ($P < 0.001$). Total cholesterol decreased after both supplements, but did not differ between the supplements. Low density lipoprotein-cholesterol did not change, and high density lipoprotein-cholesterol significantly decreased in subjects given n-3 PUFA compared to baseline, but not when compared to subjects receiving n-6 PUFA. In conclusion, we have shown that a single very high dose of n-3 PUFA has a pronounced hypotriglyceridemic effect, which is directly related to the initial plasma level.

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Fish oils rich in n-3 polyunsaturated fatty acids (PUFA) have been shown to lower plasma triglycerides in normo- and hypertriglyceridemic subjects (1–4) in a dose-dependent fashion (5). It has also been shown that the presence of n-3 PUFA in the diet reduces the postprandial lipemia 1–8 h after an oral fat load (6–9). The reduction in plasma triglycerides is most likely caused by an inhibition of very low density lipoprotein (VLDL) production in the liver (1). Even a moderate intake (5 g/d for 6 wk) of n-3 PUFA was recently shown to attenuate the lipemic response to a standard fat meal (10). In contrast, studies examining the effect of an acute load of n-3 PUFA on plasma lipids and lipoproteins in subjects on their habitual diets have given inconsistent results (6–8,11).

The present study was undertaken to investigate the effects of a single very high dose of n-3 PUFA on plasma lipids and lipoproteins 14 h after ingestion in a group of healthy subjects.

[1]This study has been presented in part as a poster at the Omega-3 Symposium, Oslo, Norway, 1992.

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Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoproteins.

TABLE 1

Composition of Fish and Vegetable Oils Used in the Study

Composition	Fish oil (%)[a]	Vegetable oil (%)[a]
Main fatty acids		
16:0	2.6	10.4
18:1	8.3	26.4
18:2	2.4	59.2
20:5	34.0	0
22:5	3.5	0
22:6	19.0	0
Total n-6	<5	61
Total n-3	68	0
Monounsaturated	17	27
Saturated	<5	12
Free fatty acids	<1	<1
Unspecified	<5	<1
Cholesterol (mg/g)	<1	1.3
Vitamin E (IU/g)	2	2

[a] % w/w of total fat.

MATERIALS AND METHODS

Patients and oil supplements. Forty apparently healthy, nonobese volunteers, who did not consume alcohol on a regular basis, were included in a randomized, double blind placebo-controlled study. Twenty females (aged 21–51 yr; median age 37.5 yr) and twenty males (aged 20–45 yr; median age 31.5 yr), all free of medication for at least 2 wk prior to the study, were included. Block randomization was used to secure an equal distribution with respect to gender. The subjects were randomly allocated to receive at the time of their evening meal at 6 p.m., either 30 capsules of a fish oil triglyceride concentrate (Epax 5500 TG; produced by Pronova Biocare a.s., Lysaker, Norway, delivered as Pikasol(R) by Lube a.s., Hadsund, Denmark) containing a total of 20 g of n-3 PUFA, or 30 capsules of corn oil (delivered by Pronova Biocare a.s.) containing 20 g of n-6 PUFA. The composition of the oil is given in Table 1.

The subjects were advised to eat their usual evening meal on the two days before blood testing, but meals containing any fish or high amounts of fat were not allowed.

Blood sampling and analyses. The subjects had blood sampled after an overnight fast resting in the supine position for 15 min at 8:00 a.m. on Day 1. Blood was drawn from an antecubital vein using minimal stasis, and the first few milliliters of blood were discarded. At 6 p.m. on the same day, the subjects took either 30 capsules of fish oil or vegetable oil with their evening meal. The next morning at 8 a.m. (14 h after ingestion of the capsules)

the subjects again had blood drawn (Day 2) using the same procedures as on Day 1. Triglyceride and total cholesterol in plasma were measured using a Cobas Mira (Roche, Switzerland). Triglycerides were measured using Triglycerides PAP(R) (Roche, Basel, Switzerland), and total cholesterol was determined using Monotest(R) (Boehringer Mannheim, Mannheim, Germany). Plasma high density lipoprotein (HDL)-cholesterol was determined after precipitation of apo B containing lipoproteins with phosphotungstic acid and magnesium. VLDL-cholesterol was determined as plasma triglycerides divided by 2.2, and low density lipoprotein (LDL)-cholesterol was calculated using the Friedewald formula (12). The study was approved by the Ethics Committee of Northern Jutland.

Statistics. Pratts test was used for comparison of results within each of the two groups. The n-3 PUFA group was compared to the n-6 PUFA (control) group using Mann-Whitney's test for unpaired data. Correlation analyses were performed by Spearman's test. A *P*-value less than 0.05 (two-tailed) was considered statistically significant.

RESULTS

Despite the large number of capsules ingested, the supplements were well tolerated. The only adverse effects noted were nausea, belching with a fishy taste and loose stools in a few of the subjects receiving fish oil.

The results on plasma lipids and lipoproteins are shown in Table 2. Total cholesterol decreased significantly after both n-3 PUFA and n-6 PUFA by 7.7 and 1.5%, respectively. The difference in the reduction between the two groups approached, but did not reach, statistical significance ($0.1 > P > 0.05$). Plasma LDL-cholesterol did not change significantly in any of the two groups, or between groups. Plasma HDL-cholesterol decreased significantly by 9.1% in the group given n-3 PUFA, whereas there was an insignificant decline in the n-6 group. However, there was no significant difference between the two supplements. VLDL-cholesterol and plasma triglycerides decreased significantly after supplementation with both n-3 PUFA and n-6 PUFA by 33 and 12%, respectively (Fig. 1). Plasma triglycerides decreased in all subjects given n-3 PUFA, and the decrease

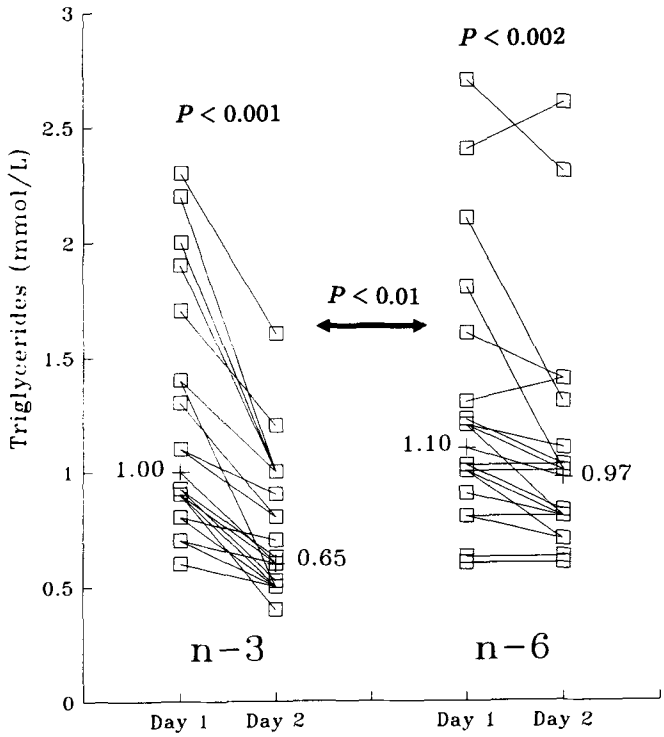

FIG. 1. The effect on plasma triglycerides of a single dose of n-3 or n-6 polyunsaturated fatty acids. Medians (+) are shown and *P*-values within and between groups are indicated.

in triglycerides was significantly greater ($P < 0.01$) in the n-3 group than in the group receiving n-6 PUFA. The hypotriglyceridemic effect in subjects receiving n-3 PUFA was significantly ($P < 0.001$) correlated to the baseline levels of plasma triglycerides, whereas such a correlation could not be demonstrated in the n-6 group.

DISCUSSION

The main finding in the present study was a decrease in plasma triglycerides in all subjects (n = 20) given n-3 PUFA with a median decrease in plasma triglycerides of 33%. This decrease was more pronounced in subjects with higher baseline levels of plasma triglycerides, as there was a significant correlation between baseline

TABLE 2

Plasma Lipids and Lipoproteins (mmol/L) Before and After Supplementation with n-3 PUFA or n-6 PUFA[a]

	n-3 PUFA		n-6 PUFA	
	Day 1	Day 2	Day 1	Day 2
Triglycerides	1.00(0.82–1.63)	0.65(0.52–0.98)[b,e]	1.10(0.92–1.52)	0.97(0.77–1.25)[c]
Total cholesterol	5.30(4.30–6.55)	4.80(3.93–6.45)[b]	6.00(5.43–6.40)	5.60(4.93–6.47)[d]
VLDL-cholesterol	0.46(0.38–0.75)	0.30(0.23–0.46)[b,e]	0.50(0.43–0.70)	0.46(0.37–0.57)[b]
LDL-cholesterol	3.05(2.35–4.30)	2.95(2.22–4.40)	3.85(3.13–4.28)	3.70(3.30–4.35)
HDL-cholesterol	1.65(1.30–1.88)	1.50(1.20–1.72)[d]	1.63(1.22–1.88)	1.53(1.23–1.88)

[a]Results are shown as medians with interquartiles (25–75%) in parentheses. PUFA, polyunsaturated fatty acids; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.
[b]$P < 0.001$.
[c]$P < 0.002$.
[d]$P < 0.05$.
[e]$P < 0.01$, comparing n-3 PUFA with n-6 PUFA.

triglyceride levels and the reduction in plasma triglycerides. Plasma levels of total cholesterol decreased after n-3 PUFA due to a reduction in HDL-cholesterol and, mainly, in VLDL cholesterol levels, while plasma LDL-cholesterol was unaltered.

While low and moderate doses of n-3 PUFA do not affect, or slightly increase, plasma HDL cholesterol, the lowering of HDL-cholesterol in the present study is in accordance with findings from studies in which n-3 PUFA were given in very high daily doses for a few weeks (1,13). The present study extends previous findings and shows that the reduction of HDL cholesterol levels by very high doses of n-3 PUFA occurs rapidly, although the mechanism by which it occurs is unknown. Thus, there is no convincing evidence that dietary n-3 PUFA decrease apolipoprotein A synthesis or enhance removal of HDL-cholesterol from the circulation. Also, studies that examined the effects of n-3 PUFA on key enzymes involved in HDL kinetics, such as cholesterol ester transfer protein and lecithin cholesterol acyltransferase, have yielded inconsistent results (14–18). It should be noted that in our study, plasma HDL-cholesterol did not significantly differ between intakes of PUFA from the n-3 *vs.* the n-6 family. Our results do not indicate, however, whether plasma triglycerides were decreased due to increased clearance of triglyceride-rich lipoproteins from the circulation or due to reduced hepatic VLDL secretion, although based on literature data, the latter may be the more likely mechanism (1,19).

The results from our study are in agreement with the findings from a recent trial in which a single dose of n-3 PUFA (50 mg/kg) was given to nine healthy subjects who had plasma triglycerides measured at baseline, and at 24, 48 and 72 h after the supplements (20). In that study, triglyceride levels significantly decreased by 15% at 24 h and 16% at 48 h, but began to normalize 72 h after n-3 PUFA intake.

The impact of treatment of hypertriglyceridemia on coronary heart disease remains controversial (21,22). However, patients with plasma levels of triglycerides above 10 mmol/L are at risk for abdominal symptoms and pancreatitis (23), and could potentially benefit from treatment with n-3 PUFA (4,13).

Fat elimination from the diet is considered the cornerstone in the treatment of patients with acute pancreatitis. However, highly concentrated preparations of n-3 PUFA could, due to their rapid lowering of plasma triglycerides and chylomicrons, be considered for study in patients with acute pancreatitis, especially as n-3 PUFA are now becoming available for intravenous infusion (24,25). Before such investigations are undertaken, the acute effects of n-3 PUFA in patients with hypertriglyceridemia should, however, be established.

In conclusion, we have shown that a single very high dose of n-3 PUFA has a pronounced hypotriglyceridemic effect in healthy subjects. The magnitude of this effect is directly related to the initial plasma triglyceride level.

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Singlet Oxygen Quenching Ability of Naturally Occurring Carotenoids

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The singlet oxygen quenching ability of various naturally occurring carotenoids was examined by measuring toluidine blue-sensitized photooxidation of linoleic acid. To assess quenching, the oxidation of linoleic acid was followed by measuring oxygen consumption and ultraviolet absorbance at 235 nm. We found that oxygen quenching increased as the number of conjugated double bonds in the carotenoids increased, but quenching varied with chain structure and functional groups. Acyclic carotenoids enhanced quenching more than did cyclic carotenoids. Conjugated keto groups and the presence of a cyclopentane ring stimulated quenching, while hydroxy, epoxy and methoxy groups showed lesser effects. The photosynthetic bacterial carotenoids, spirilloxanthin and rhodopin, were found to be most effective as quenchers, followed by the cayenne carotenoid, capsorbin.
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There is currently much interest in the role of active oxygen and free radicals in disease processes. It is being realized that active oxygen species, such as superoxide anion, hydrogen peroxide and singlet oxygen, can induce destructive events, including enzyme inactivation, DNA-strand cleavage and membrane lipid peroxidation (1–3).

A number of studies have indicated that ß-carotene is able to quench singlet oxygen (4) and oxygen free radicals (5). Earlier research on carotenoid function was mostly focussed on ß-carotene. Recently, several other carotenoids and xanthophylls were examined, and lycopene and astaxanthin were reported to be more effective antioxidants than ß-carotene (6,7).

In the present study we investigated the relative singlet oxygen quenching abilities of several additional naturally occurring carotenoids. Quenching was measured by monitoring toluidine blue-sensitized photooxidation of linoleic acid. The relationship between structure and quenching ability of the carotenoids will be discussed.

MATERIALS AND METHODS

Nineteen carotenoids were prepared from higher plants, animals, marine algae and photosynthetic bacteria (PSB) according to the published methods (8,9). A simple and rapid method for the measurement of linoleic acid photooxidation was employed, using toluidine blue as the photosensitizing dye. In a micro glass vial (2 mL), toluidine blue (final concentration, 0.05 mM) and linoleic acid (final concentration, 60 mM) with or without carotenoids (final concentration, 15 µM) were added, and the final volume was adjusted to 1 mL with *n*-hexane/ethanol (1:1, vol/vol). The vial was tightly closed with a screw cap that had a septum. The mixture was illuminated at 11,000 lux at 30°C for 2 h in a photochamber (LX-3000, Taitec Co., Saitama, Japan). After completion of the reaction, 100 µL of headspace gas was removed with a microsyringe and gas chromatographed on a molecular sieve 13X column to estimate oxygen consumption. In addition, 100 µL of the reaction mixture was removed and diluted to 3 mL with ethanol, and the absorbance at 235 nm was measured to estimate the formation of conjugated dienes.

RESULTS AND DISCUSSION

Table 1 shows the inhibitory effects of various naturally occurring carotenoids on photooxidation of linoleic acid, which were measured by monitoring oxygen consumption and ultraviolet absorbance at 235 nm (conjugated diene formation). Based on the oxygen consumption rate in the reaction, it was found that the quenching ability varied greatly, depending on the structures of carotenoids. As reported previously (10,11), the number of conjugated double bonds is the most effective parameter to assess singlet oxygen quenching ability. Comparison of the structure and quenching ability of ß-carotene with those of lycopene, and those of cryptoxanthin and rhodopin, indicates that an open chain has a higher quenching ability than a ß-ionone ring. Smaller differences between the abilities of ß-carotene and cryptoxanin, and of spheroidene and OH-spheroidene, suggest that the hydroxy group has a positive effect but is less effective as quencher. Similarly, epoxy and methoxy groups have lesser effects. The effects of capsanthin and capsorbin were more pronounced than those of ß-carotene and cryptoxanthin, although they have the same number of conjugated double bonds. This higher potency could be due to the presence of conjugated keto groups and of the cyclopentane rings. Isolated C=C double bonds seem to have an additional effect on quenching. The relatively higher potency of lycopene than of other carotenoids with the same number of conjugated double bonds can probably be attributed to the presence of the two isolated double bonds and an acyclic chain effect.

The oxidation of linoleic acid by singlet oxygen is expected to result in roughly half the products consisting of conjugated diene hydroperoxides, and half being nonconjugated. The pattern of conjugated diene formation measured by absorbance at 235 nm was similar to that of O_2 consumption rates, suggesting that absorbance at 235 nm could be used as index for singlet oxygen-quenching by carotenoids.

It is of interest that photosynthetic bacterial cells have a large amount of highly effective acyclic carotenoids, such as spirilloxanthin, rhodopin, anhydrorhodovibrin and lycopene. The observed therapeutic effects of PSB cells on growing young fish (12) may be related, in part, to oxygen quenching by carotenoids. The

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Abbreviation: PSB, photosynthetic bacteria.

TABLE 1

Inhibitory Effects of Various Carotenoids on Toluidine Blue-Sensitized Photooxidation of Linoleic Acid

Carotenoids	Sources	F[a]	Carbon chain	Functional groups[b]	O_2 consumption		Conjugated diene formation	
					μmol[c]	%[d]	A_{235}[c]	%[d]
None					1.980	100	0.845	100
Neurosporen	PSB	9	acyclic		1.340	68	0.681	81
Violaxanthin	Spinach	9	cyclic	2OH, 2O<	0.833	42	0.336	40
Neoxanthin	Spinach	10[e]	cyclic	3OH, O<	1.299	66	0.525	62
Fucoxanthin	Brown algae	10[e]	cyclic	2OH CO[f], O< CH_3CO	1.110	56	0.423	50
Spheroidene	PSB	10	acyclic	CH_3O	0.931	47	0.353	42
OH-spheroidene	PSB	10	acyclic	OH, SH_3O	0.803	41	0.298	35
Lutein	Spinach	10	cyclic	2OH	0.970	49	0.423	50
β-Carotene	Spinach	11	cyclic		1.064	54	0.468	55
Cryptoxanthin	Persinon	11	cyclic	OH	0.990	50	0.439	52
Spheroidenone	PSB	11	acyclic	CH_3O, CO[f]	0.772	39	0.338	40
OH-spheroidenone	PSB	11	acyclic	OH, CH_3O, CO[f]	0.738	37	0.323	38
Capsanthin	Cayenne	11	cyclic	2OH, CO[f]	0.666	34	0.258	31
Lycopene	Tomato	11(2)	acyclic		0.621	30	0.288	34
Capsorbin	Cayenne	11	cyclic	2OH, 2CO[f]	0.535	27	0.236	28
Rhodopin	PSB	11(1)	acyclic	OH	0.511	26	0.219	26
Anhydrorhodovibrin	PSB	12	acyclic	CH_3O	0.597	30	0.217	26
Astaxanthin	Salmon	13	cyclic	2OH, 2CO[f]	0.719	36	0.311	37
Spirilloxanthin	PSB	13	acyclic	$2CH_3O$	0.413	21	0.187	22

[a]Number of conjugated double bonds; the number in parentheses gives isolated double bonds.

[b]OH, hydroxy; O<, epoxy; CO, carbonyl; CH_3CO, acetyl; CH_3O, methoxy; PSB, photosynthetic bacteria.

[c]The values were obtained for photooxidation of linoleic acid at 30°C for 2 h.

[d]Percent control (none).

[e]C=C=C group as conjugated double bonds.

[f]Conjugated keto group.

highly effective carotenoids of cayenne, capsunthin and capsorbin, equally attract attention as effective oxygen quenchers.

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ARTICLE

Inhibition of Human Neutrophil Leukotriene B_4 Synthesis in Essential Fatty Acid Deficiency: Role of Leukotriene A Hydrolase

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A female subject dependent on long-term total parenteral nutrition developed an aversion and noncompliance to a prescribed weekly lipid infusion designed to meet essential fatty acid (EFA) requirements. Fatty acids (FA) in the subject's plasma and isolated peripheral blood neutrophils were analyzed in search of biochemical evidence of EFA deficiency. Neutrophil 5-lipoxygenase metabolism was examined to assess the possible effects of EFA deficiency on neutrophil eicosanoid metabolism. EFA deficiency was confirmed by marked depletion of linoleic acid (18:2n-6) and accumulation of eicosatrienoic acid (ETrA; 20:3n-9) in plasma and neutrophil phospholipids. In the neutrophils, ETrA comprised 5.2% of phospholipid FA (normal reference values <0.1%), and arachidonic acid (AA; 20:4n-6) comprised 8.6% of phospholipid FA (normal reference range 10–16%). When stimulated by A23187 *in vitro* on three separate occasions, the subject's neutrophils displayed impaired synthesis of leukotriene B_4 (LTB_4), but produced normal amounts of 5-hydroxyeicosatetraenoic acid and *all-trans* isomers of LTB_4 formed nonenzymatically from leukotriene A_4 (LTA_4). This pattern of synthesis suggested inhibition of LTA hydrolase and was also seen in neutrophils from healthy subjects by addition of exogenous ETrA *in vitro*. Comparative studies of the effects of ETrA and eico-sapentaenoic acid (20:5n-3) on neutrophils *in vitro* suggested that ETrA is the more potent inhibitor. Accumulation of ETrA, rather than depletion of AA, appears principally responsible for the observed impairment of neutrophil LTB_4 synthesis seen in this EFA-deficient subject.
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Eicosanoid mediators of inflammation, such as prostaglandins, thromboxane and leukotrienes are typically derived from arachidonic acid (AA, 20:4n-6) as n-6 fatty acids (FA) are the prevalent essential fatty acids (EFA) in the Western diet, which contains relatively little n-3 FA. In the presence of severe dietary EFA deficiency, oleic acid (18:1n-9), which can be synthesized endogenously, becomes the predominant substrate for the hepatic desaturase/elongase enzymes that normally convert dietary linoleic acid (LA; 18:2n-6) to AA. As a result, plasma and cellular levels of eicosatrienoic acid (ETrA; 20:3n-9) increase, and the overall cellular content of 20 carbon highly unsaturated fatty acids is maintained, in spite of AA depletion (1,2).

EFA deficiency has been associated with reduced inflammatory responses in animals (3,4), including amelioration in models of inflammatory disease (5–8). Early studies demonstrated that EFA-deficient animals with experimentally-induced inflammation had reduced production of prostaglandins derived from AA *via* the cyclooxygenase pathway (3). Prostaglandins can have ambivalent effects on inflammation, including mediation of enhanced nociception (8) and tissue oedema (9,10), but decreased T lymphocyte responses (11).

More recently, the potential importance of the alternate 5-lipoxygenase pathway of AA metabolism has been recognized. This pathway is active in both mononuclear phagocytes and neutrophils and, in the latter, is the principal pathway of AA metabolism (12). An important product of this pathway is leukotriene B_4 (LTB_4), which is a very potent neutrophil chemotaxin and also stimulates neutrophil degranulation and oxidative burst activity (12). LTB_4 can have pro-inflammatory effects on mononuclear cells, including enhancement of production of the key inflammatory cytokines interleukin-1 (13,14), interleukin-2 (15) and interferon-gamma (15,16). LTB_4 inhibits mitogen-induced proliferation of $CD4^+$ T cells and enhances proliferation of $CD8^+$ T cells (17,18) and, accordingly, may also modulate immunological responses. EFA deficiency in rats is associated with marked inhibition of LTB_4 production by neutrophils stimulated *in vivo* (19,20). The effect of EFA deficiency on LTB_4 production in humans has not been reported.

A woman receiving total parenteral nutrition with intolerance to intravenous lipid therapy and severe EFA deficiency has provided the opportunity to assess this issue in a human subject. *In vitro* studies, in which neutrophils from healthy volunteers were treated with ETrA, were also undertaken to assess the possible relationship between the observed ETrA accumulation and impaired LTB_4 synthesis in the neutrophils of the EFA-deficient subject.

MATERIALS AND METHODS

Experimental subject. A 33-year-old female had been maintained on total parenteral nutrition *via* an indwelling subclavian catheter for 12 years, following subtotal resection of the small and large bowel for idiopathic intestinal pseudo-obstruction. She also had three successful pregnancies during this period. Complications included superior vena cava obstruction, recurrent septicaemia, proximal myopathy and an osteoporotic rib

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Abbreviations: AA, arachidonic acid (20:4n-6); EFA, essential fatty acids; EPA, eicosapentaenoic acid; ETrA, n-9 eicosatrienoic acid (20:3n-9); FA, fatty acid(s); HPLC, high-performance liquid chromatography; LA, linoleic acid (18:2n-6); LTA, leukotriene A; LTB_4, leukotriene B_4; 5-HETE, 5-hydroxyeicosatetraenoic acid.

fracture. Typically, the intravenous regimen involved daily 1800-mL infusions containing glucose, amino acids and electrolytes (Vamin or Synthamin; Baxter Health Care, Sydney, NSW, Australia). MVI-12 (Rhone-Poulenc, Melbourne, Victoria, Australia) was given daily to provide water-soluble vitamins (one 5-mL vial) and fat-soluble vitamins (one 5-mL vial). Intralipid 20% (Baxter Health Care), 500 mL weekly, was prescribed as the source of dietary fat, including EFA. Due to intolerance of the fat infusion, the interval of self-administration was typically two weeks, and the infusion was not completed. Concentrations of fat soluble vitamins in plasma were as follows: vitamin A, 0.9 mmol/L (R 2.1–6.7); vitamin E, 10.5 mmol/L (R 12–46) and vitamin D, 39 nmol/L (R 40–160). Vitamin K was not measured because the subject was receiving warfarin treatment. Forearm bone content was between the first and tenth percentiles with bone mineral content 0.86 g/cm (fat corrected to 0.91) and bone mineral density 344 mg/mL (expected 426). The selenium concentration was 1 mmol/L (normal range >1) and zinc 14.2 mmol/L (R 9–18). Total carnitine was 13 mmol/L (R 35–65) with free carnitine 12 mmol/L (R 30–60), acylated carnitine 1 mmol/L and the ratio of acylated to free 0.08 (R 0.10–0.75). *In vitro* tests of neutrophil function revealed normal degranulation, oxidative burst and bactericidal activity.

Healthy subjects. Peripheral venous blood samples from healthy subjects were used to establish reference ranges for analysis of (i) fatty acids in plasma (n = 40) and neutrophil phospholipid (n = 29) and (ii) neutrophil 5-lipoxygenase pathway responses (n = 44). No difference was seen between males and females for any of these analyses. Healthy laboratory personnel (one male, two female) provided blood samples for studies on the effects of exogenous fatty acids added to neutrophils *in vitro* prior to cell stimulation.

FA analysis. Neutrophil phospholipid fractions were isolated by chloroform/methanol extraction and thin-layer chromatography. The phospholipids were then subjected to acid methanolysis by using 1% H_2SO_4 in methanol at 70°C for 3 h. Fatty acid methyl esters were resolved and quantified by capillary gas–liquid chromatography on 50 M WCOT columns coated with SP2330 (Chromalytic Technology, Boronia, Victoria, Australia), as described previously (21). All organic solvents contained 0.005% butylated hydroxyanisole. The analysis allowed quantification of saturated and unsaturated fatty acids, including n-7 fatty acids, which are increased in EFA deficiency.

Leukotriene and 5-hydroxy acid production. Peripheral blood neutrophils were isolated using a double noncontinuous Percoll gradient, as previously described (22). Neutrophils (10^6/mL) were stimulated with A23187 (0.5 µmol/L final concentration, Sigma Chemical Co., St. Louis, MO). After 5 min at 37°C, the mixture was acidified with 0.25 mL of 100 mM citric acid; 15-hydroxyeicosatetraenoic acid (15-HETE) (Cayman Chemical, Ann Arbor, MI) was added as an internal standard. Leukotrienes and hydroxy acids, including 5-HETE and the *all-trans* isomers of LTB_4, were assayed by high-performance liquid chromatography as previously described (23). 20-Hydroxy LTB_4, formed by ω-oxidation of LTB_4, was also measured. Under the stimulation conditions used, 20-carboxy LTB_4 constituted 13% (±2%) of the total ω-oxidation products, and as the low values were frequently near the limits of detection, the values for 20-carboxy LTB_4 were not compiled.

To assess the effects of exogenous FA added *in vitro*, ETrA (Cayman Chemical) or eicosapentaenoic acid (EPA) (Sigma Chemical Co.) were added to neutrophils from healthy volunteers (10^6/mL; final concentration of fatty acids, 1–5 µmol/L; methanol, 0.05%) followed by pre-incubation at 25°C (5 min) prior to stimulation with A23187, as described in the previous paragraph.

RESULTS

Plasma and leukocyte FA. Analysis of FAs in the experimental subject's plasma revealed a triene/tetraene (ETrA/AA) ratio of 4.3. A ratio of greater than 0.2 indicates EFA deficiency (1). Accumulation of n-7 and n-9 FAs, including ETrA, was seen in total plasma FA and neutrophil phospholipids. In the leukocyte phospholipids, ETrA constituted 5.2% of total FA, and AA was reduced compared with levels found in leukocytes from healthy subjects (Table 1). These data indicate severe EFA deficiency.

Effect of EFA deficiency on neutrophil LTB_4 synthetic pathway. Neutrophil LTB_4 production, as assessed by assaying LTB_4 and 20-hydroxy LTB_4, was reduced to more than two standard deviations below the mean for healthy subjects (Fig. 1). 5-HETE and the *all-trans* isomers of LTB_4 (LTB_4 isomers) were not reduced (Fig. 1). The ratio of (LTB_4 + 20-hydroxy LTB_4)/(LTB_4 + 20-hydroxy LTB_4 + LTB_4 isomers), which is an index of the fraction of available leukotriene A_4 (LTA_4) converted by LTA hydrolase to LTB_4, was reduced compared to the normal range for healthy subjects (Fig. 2). The observed

TABLE 1

Fatty Acid Composition of Total Plasma Lipids and Peripheral Blood Neutrophil Phospholipids from a Female Subject Dependent on Chronic Total Parenteral Nutrition[a]

	Fatty acids (% total fatty acids)			
	Total plasma		Neutrophil phospholipids	
	Observed	Ref. range	Observed	Ref. range
16:1n-7	6.5	0.7–3.5	1.4	<0.1–0.8
18:1n-7	5.5	1.0–2.8	5.7	0.8–1.6
Total n-7	12.0	1.8–5.8	7.1	0.5–2.1
18:1n-9	36.2	15–28	33.4	23–29
20:3n-9	11.5	<0.1–0.4	5.2	<0.1
Total n-9	51.5	16–29	44.1	24–31
18:2n-6	0.6	21–38	0.7	7–15
20:4n-6	2.7	3.2–9.6	8.6	10–16
Total n-6	4.3	29–48	10.9	25–34
Total n-3	0.6	1.3–5.3	1.1	1.4–6.2

[a]Reference (Ref.) ranges were established using samples from healthy subjects (plasma, n = 40; neutrophil phospholipids, n = 29). The range shown is from two standard deviations above and below the mean for healthy subjects.

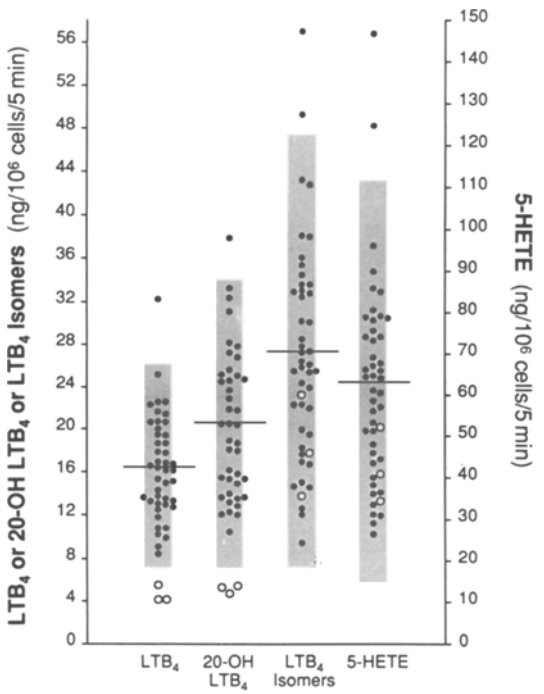

FIG. 1. Production of leukotriene B_4 (LTB_4) 20-hydroxy LTB_4, LTB_4 isomers and 5-hydroxyeicosatetraenoic acid (5-HETE) by neutrophils stimulated by A23187, as described in the Materials and Methods section. Results from the essential fatty acid-deficient subject's neutrophils studied on three separate occasions (open circles) and neutrophils from healthy volunteers (closed circles) are shown. The shaded areas indicate two standard deviations above and below the mean (horizontal lines) for healthy subjects.

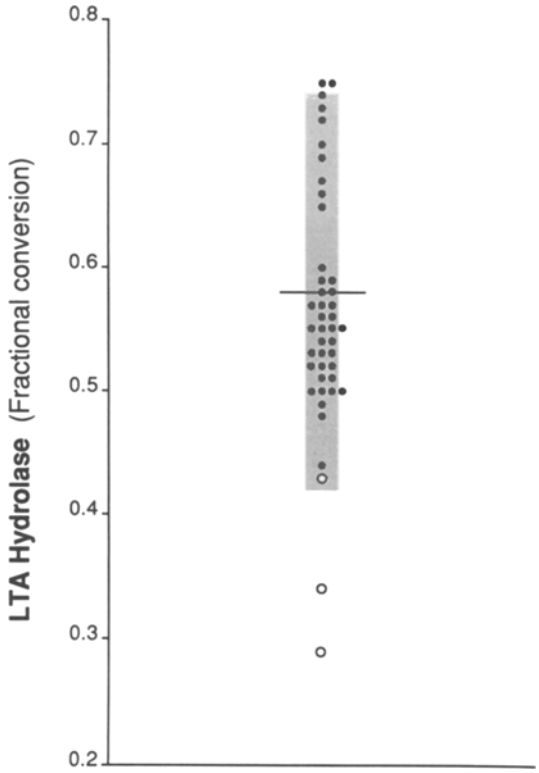

FIG. 2. Leukotriene A (LTA) hydrolase fractional conversion is computed as (LTB_4 + 20-hydroxy LTB_4)/(LTB_4 + 20-hydroxy LTB_4 + LTB_4 isomers). The denominator reflects the substrate potentially available to LTA hydrolase for enzymatic conversion to LTB_4. Open circles indicate values obtained with neutrophils isolated from the essential fatty acid-deficient subject on three separate occasions. Closed circles indicate values obtained with neutrophils from healthy subjects. The data used for these computations are those shown in Figure 1. The shaded areas indicate two standard deviations above and below the mean (horizontal line) for healthy subjects. LTB, leukotriene B.

normal production of 5-HETE and LTB_4 isomers suggests normal availability of AA for metabolism by 5-lipoxygenase, whereas reduced fractional conversion of LTA_4 to LTB_4 suggests inhibition of LTA hydrolase.

LTB_4 had a retention time of 11.6 min. Dual high-performance liquid chromatography (HPLC) peaks at slightly longer retention times were observed with ratios of retention times relative to LTB_4 of 1.48 and 1.29. These peaks were not observed in control subjects.

Correction of EFA deficiency. Once it was established that the subject was EFA deficient, the following adjustments were made to the regimen for lipid infusion. Intralipid (500 mL) was diluted with dextrose solution (50%, 500 mL) and an electrolyte solution (Synthamin 17, 750 mL) prior to infusion, and the infusion was carried out over a longer period of time. With these adjustments, the subject was able to complete the weekly lipid infusion without significant unwanted effects. After approximately two years on this revised regimen, neutrophil fatty acid values were: LA, 6.2%; ETrA, 0.5%; and AA, 12.4%. Rates of synthesis of 5-lipoxygenase products were as follows (ng/10^6 cells/5 min): LTB_4, 18.5; 20-hydroxy LTB_4, 17.8; 5-HETE, 53.3; *all-trans* isomers of LTB_4, 19.6 (the normal reference ranges are shown in Fig. 1).

Effect of exogenous ETrA on neutrophil LTB synthetic pathway. Addition of ETrA *in vitro* to neutrophils from

TABLE 2

Effect on Neutrophil Leukotriene B_4 (LTB_4) Production of Exogenous Eicosapentaenoic Acid (EPA) and n-9 Eicosatrienoic Acid (ETrA) Added Alone and in Combination[a]

Fatty acid (μmol/L		LTB_4 synthesis (mean ± SEM; ng/10^6 cells/5 min)
EPA	ETrA	
0	0	14.2 ± 0.4
1	0	13.5 ± 0.1
0	1	11.2 ± 0.6
1	1	9.8 ± 0.6
2.5	0	9.2 ± 0.5
0	2.5	7.4 ± 0.3
2.5	2.5	6.2 ± 0.5

[a]Human neutrophils from a healthy subject were pre-incubated with fatty acids and stimulated with A23187 as described in the Materials and Methods section. Incubations were performed in triplicate.

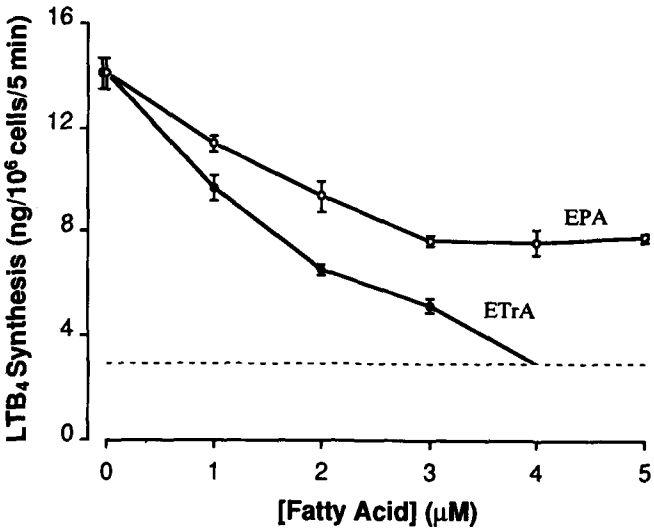

FIG. 3. Effect of exogenous eicosapentaenoic acid (EPA) (open circles) and eicosatrienoic acid (ETrA) (closed circles) on LTB_4 production by human neutrophils in response to A23187 under conditions described in the Materials and Methods section. Results are expressed as mean ± SEM of triplicate incubations. The horizontal dashed line represents the minimum detectable amount. Data shown are with neutrophils isolated from a single healthy subject. Similar findings were obtained with neutrophils from two additional subjects (data not shown). See Figure 2 for other abbreviations.

three healthy subjects reproduced the pattern of hydroxy acid synthesis observed in the EFA-deficient subject, *viz.*, reduced production of LTB_4 (Fig. 3) and 20-hydroxy LTB_4, but not LTB_4 isomers (data not shown). Thus the pattern of neutrophil LTA hydrolase inhibition found in the EFA-deficient subject was induced in normal neutrophils by addition of ETrA *in vitro*. Dual peaks with ratios of retention times relative to that of the LTB_4 peak of 1.50 and 1.30 were observed in chromatograms from ETrA-treated, but not control, cell preparations.

Comparison of effects of ETrA and EPA. Exogenous ETrA was a more potent inhibitor of LTB_4 production than exogenous EPA (Fig. 3 and Table 2). Additive inhibitory effects of EPA and ETrA added *in vitro* on neutrophil LTB_4 production were seen (Table 2).

DISCUSSION

The altered pattern of synthesis of 5-lipoxygenase products seen in this EFA-deficient subject is consistent with inhibition of LTA hydrolase (24–26). The appearance of dual HPLC peaks with retention times slightly greater than that of LTB_4 when neutrophils were treated with ETrA, is consistent with the formation of the *all-trans* isomers of LTB_3, which are nonenzymatic breakdowns of LTA_3 and which are resolved in this manner relative to LTB_4 (24). Whatever the identity of these peaks, their presence in the chromatogram from the EFA-deficient subject suggests that ETrA formed *in vivo* was metabolized by the EFA-deficient neutrophils. Dietary supplementation with EPA-rich fish oils and addition of exogenous EPA to neutrophils *in vitro* yielded a similar alteration in n-6 products (27,28), which was attributed to inhibition of LTA hydrolase by LTA_5 derived from EPA (29). Both ETrA and EPA can be converted to products, i.e., LTA_3 and LTA_5, respectively, which are less efficiently metabolized by LTA hydrolase than LTA_4 derived from AA, but which can act as competitive inhibitors of LTB_4 production. Insignificant amounts of LTA_3 are converted to LTB_3 (26). Although LTB_5 is formed in measurable amounts, this compound has much less chemotactic activity than does LTB_4 (30–32).

Dietary fish oil supplements containing EPA lessen symptoms in rheumatoid arthritis and reduce neutrophil LTB_4 synthesis *ex vivo* (27,28,33,34). However, it has been reported that suppression of LTB_4 production is greater in EFA-deficient rats than in fish oil-supplemented rats (20). EFA deficiency has been consistently associated with reduced inflammation and reduced organ damage in animal models of inflammation (3–6). Although no direct experimental comparisons have been reported, in small laboratory rodents, EFA deficiency seems to be associated with suppression of inflammation to a degree at least as great as that achieved with fish oil diets. Indeed, fish oil treatment can have ambiguous effects in animal models of inflammation (35–37) and has yielded only modest symptomatic improvement in patients with rheumatoid arthritis (27,28,33,34) and psoriasis (38,39). This lack of potency may, in part, relate to the ambivalent effects of EPA on production of inflammatory mediators. For example, inhibition of LTB_4 production potentially reduces leukocyte recruitment and degranulation (12), whereas alteration in prostacyclin/thromboxane balance in favor of the vasodilatory effects of prostacyclin could increase tissue oedema (8,9). Unlike AA and EPA, ETrA cannot be metabolized to prostacyclin or other ring structure prostaglandins that might potentiate tissue oedema, but it does inhibit LTB_4 production and thus might be expected to exert a more consistent anti-inflammatory effect than EPA.

When normal dietary requirements for LA are met, there is very little conversion of the 18 carbon fatty acids of the n-3 and the n-9 series to their respective 20 carbon fatty acids, EPA (n-3) and ETrA (n-9) (40,41). The present case indicates that EFA deficiency can be relatively well tolerated, as the subject accepted the dryness and scaliness of her skin as preferable to the unpleasant effects of bolus therapy with Intralipid. However, avoidance of dietary LA sufficient to allow accumulation of ETrA is not a realistic approach to the treatment of inflammatory diseases in free-living human subjects. Direct fortification of the diet with ETrA could provide a more practical strategy.

The n-3 analogue of ETrA, EPA can be incorporated into cell membranes in competition with n-6 fats when introduced directly within an EFA-sufficient diet, as evinced by numerous animal and human intervention studies (42–44). Similarly, ETrA was incorporated into the cell membranes of rabbits when fed together with an EFA-sufficient diet, with a broadly similar efficiency to that reported for EPA (Gibson, R.A., and Neumann,

M.A., unpublished results). Thus, dietary supplementation with ETrA could conceivably result in accumulation of ETrA in cell membranes sufficient to influence synthesis of inflammatory eicosanoids.

Cold water fish are rich in n-3 fats and fish oils, and extracts of cultures of certain marine bacteria and yeasts (45,46) provide commercially available sources of concentrated EPA for dietary supplementation. The lack of a similar high yield biological source of ETrA or a cheap synthetic process is an obstacle to the evaluation of the possible anti-inflammatory effects of ETrA. Because ETrA lacks the unpleasant odor of EPA-rich marine oil preparations and is chemically more stable than EPA, because it is less unsaturated, it could be integrated into foods. Such an approach should be far more acceptable to consumers than the strategy of giving fish oils in gelatin capsules. This advantage, plus the effect of ETrA on LTA hydrolase, which can be the rate-limiting enzyme in the LTB_4 synthetic pathway (47), justifies further investigations into the anti-inflammatory effects of ETrA and biosynthetic sources for its production.

ACKNOWLEDGMENTS

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The Effect of Linoleic, Arachidonic and Eicosapentaenoic Acid Supplementation on Prostacyclin Production in Rats

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We examined the effect of dietary supplementation of linoleic acid (LA), arachidonic acid (AA) or eicosapentaenoic acid (EPA) to rats fed a diet low in linoleic acid on *in vitro* and *in vivo* production of prostacyclin. Male Sprague Dawley rats were fed a high-fat diet (50% energy as fat, 1.5% linoleic acid) for two weeks. Three of the groups were then supplemented orally with either 90 mg/d of LA, AA or EPA, all as the ethyl esters, for a further two weeks while remaining on the high-fat diet. Forty-eight hour urine samples were collected at the end of the second and fourth weeks. *In vivo* prostacyclin production was determined by a stable isotope dilution, gas chromatography/mass spectrometry assay for the major urinary metabolite of prostacyclins (2,3-dinor-6-keto-$PGF_{1\alpha}$ or PGI_2-M and Δ^{17}-2,3-dinor-6-keto-$PGF_{1\alpha}$ or PGI_3-M). *In vitro* prostacyclin production was determined by radioimmunoassay of the stable metabolite (6-keto-$PGF_{1\alpha}$) following incubation of arterial tissue. Oral supplementation with AA resulted in a rise in plasma and aorta 20:4n-6, and increased *in vitro* prostacyclin and urinary PGI_2-M production. EPA supplementation resulted in a rise in plasma and aorta 20:5n-3 and 22:5n-3, and a decline in plasma 20:4n-6, but not in the aorta. In the EPA-supplemented group, the *in vitro* prostacyclin and the urinary PGI_3-M increased, but urinary PGI_2-M decreased. The increase in *in vitro* prostacyclin production in the EPA-supplemented rats was unexpected and without obvious explanation. Supplementation with LA had minimal effect on fatty acid composition of plasma or aorta and caused no change in prostacyclin production with either method. The *in vivo* measure of prostacyclin production was positively correlated with aorta AA levels, and negatively correlated with aorta levels of EPA. There was a significant positive correlation between the *in vitro* production of prostacyclin and the *in vivo* production (as measured by the urinary prostacyclin metabolite level), despite the differences observed in the EPA-fed group. There was a high inter-animal variability in prostacyclin production using either method. These results indicate that dietary AA stimulates and dietary EPA reduces *in vivo* PGI_2 production in the rat. An equivalent amount of dietary LA was without effect.
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We have previously shown that when rats were fed high-fat diets low in linoleic acid [1.2–1.5% linoleic acid (LA)] with a relatively low n-6/n-3 polyunsaturated fatty acids (PUFA) ratio (as either beef tallow or butter) there was a decrease in arachidonic acid (AA) and an increase in eicosapentaenoic acid (EPA) in phospholipids (PL) of plasma, platelets and aorta of the rats compared with rats fed low-fat diets (1-4). These fatty acid changes were associated with a decrease in the *in vitro* arterial prostacyclin production and collagen-induced thromboxane production [as measured by radioimmunoassay (RIA) of their stable metabolites] (1–4). In subsequent studies, we showed that when rats fed high-fat diets (containing butter) were supplemented with 80 mg/d of AA for 2 wk there was complete restoration of tissue AA levels and also of the *in vitro* levels of prostacyclin and thromboxane production (5).

Therefore, the *in vitro* prostacyclin and thromboxane production appeared to be regulated either directly by tissue levels of their precursor AA or indirectly by levels of EPA and docosahexaenoic acid (DHA, 22:6n-3), which may competitively inhibit AA metabolism *via* the cyclooxygenase pathway (6), or a combination of both. In these studies, the relative importance of decreased AA levels or increased EPA levels on eicosanoid synthesis in the rat has not been established.

In vitro methods for determining eicosanoids are subject to some major limitations and the aim of this study was to compare the *in vitro* method for prostacyclin used in our previous studies with an *in vivo* assessment of prostacyclin. With the *in vitro* method used, there is a propensity to produce artifactual results due to *ex vivo* activation of arterial endothelium during sampling. Prostacyclin I_2 (PGI_2) is rapidly converted to its nonenzymatic hydrolysis product 6-keto-$PGF_{1\alpha}$ under physiological conditions (7), and it is this primary metabolite which is assayed by the *in vitro* method. Secondly, it is unlikely that the *in vitro* RIA assay for prostacyclin distinguishes between the metabolite derived from PGI_2 (6-keto-$PGF_{1\alpha}$) and that derived from prostacyclin I_3 (PGI_3, Δ^{17}-6-keto-$PGF_{1\alpha}$). Since both sets of metabolites are potentially produced in rats with a low AA/EPA ratio in endothelial cells (such as in our previous studies), the interpretation of RIA results could be ambiguous.

These problems can be overcome by the measurement of the systemic production of prostacyclin *in vivo* by quantification of the characteristic metabolites of prostacyclin in urine (8). In humans (9,10) and rats (11) the ß-oxidized metabolite of PGI_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ is the major metabolite excreted in urine, whereas 6-keto-$PGF_{1\alpha}$ mainly reflects renal synthesis (12). With the application of combined gas chromatography/mass spectrometry (GC/MS) to the identification and quantification of urinary metabolites of prostaglandins, an accurate assessment of systemic PGI_2 production has been made possible. Thus potential artifacts related to sampling and handling of arterial tissue are

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Abbreviations: AA, arachidonic acid (20:4n-6); BSTFA, *N,N-bis*(trimethylsilyl) trifluoroacetamide; DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); GC/MS, gas chromatography/mass spectrometry; KRB, Krebs Ringer bicarbonate buffer; LA, linoleic acid (18:2n-6); NICI, negative ion chemical ionization; PGI, prostaglandin I (prostacyclin); PGI_2-M, 2,3-dinor-6-keto-$PGF_{1\alpha}$; PGI_3-M, Δ^{17}-2,3-dinor-6-keto-$PGF_{1\alpha}$; PL, phospholipid; PUFA, polyunsaturated fatty acid; RIA, radioimmunoassay; SIM, selected ion monitoring; TLC, thin-layer chromatography.

avoided and the exact identity of the metabolite being measured is known.

The aims of the present study were (i) to determine the effect of feeding LA, AA or EPA on *in vivo* prostacyclin production in the rat and (ii) to compare the standard *in vitro* RIA method for the determination of prostacyclin production with the *in vivo* GC/MS urinary assay for prostacyclin metabolites.

MATERIALS AND METHODS

Materials. Sodium thiopentone was purchased from Abbott Australasia (Sydney, Australia). Scintillation proximity assay RIA kits for 6-keto-$PGF_{1\alpha}$ were purchased from Amersham Australia Pty Ltd. (Sydney, Australia). Standard 2,3-dinor-6-keto-$PGF_{1\alpha}$ was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Tetradeuterated [19,19,20,20-$^2H_4$]2,3-dinor-6-keto $PGF_{1\alpha}$ was a generous gift from Upjohn (Kalamazoo, MI). *N,N-bis*(trimethylsilyl) trifluoroacetamide (BSTFA), pentafluorobenzyl bromide, methoxylamine hydrochloride and triethylamine were purchased from Pierce (Rockford, IL). "Clinelute" disposable columns (CE 1005) used for urinary extraction were purchased from Analytichem International (Harbor City, CA). All solvents were high purity grade (Suprapur) supplied by BDH-Merck (Poole, England), unless otherwise indicated. Diisopropylamine was supplied by the Aldrich Chemical Company (Milwaukee, WI). Ethyl arachidonate and ethyl linoleate were supplied by Nu-Chek-Prep (Elysian, MN). Ethyl eicosapentaenoate was purchased from Marubeni Corporation (Tokyo, Japan). Thin-layer chromatography (TLC) was performed on four-channel Silica Gel 60 A (LK6DF) plates from Whatman International (Maidstone, England). Metabolic cages were supplied by IFFA Credo (Domaine des Oncins, France). Disposable polypropylene tubes were supplied by Sarstedt (Rommelsdorf, Germany). BP-1 capillary columns were purchased from SGE (Melbourne, Australia), and CP-SIL 88 capillary columns from Chrompak (Middelburg, The Netherlands). Diheptadecanoyl phosphatidylcholine was supplied by Sigma Chemical Co. (St. Louis, MO).

Animals. Forty male Sprague Dawley rats (140–220 g) were housed in a temperature-controlled room with a 12-h light-dark cycle. Ten rats were randomly allocated to each of four dietary groups. Food and water were provided *ad libitum* and food consumption and body weight recorded three times per week. Rats were placed in metabolic cages on days 1, 14 and 28 and urine collected for 48 h into containers packed in dry ice. The urine was then stored at -80°C for later extraction of the prostacyclin metabolite and its subsequent quantification by GC/MS.

Diets. The nutrient composition of the high-fat diet per 100 g diet was 29.5 g corn starch, 18.9 g casein, 18.9 g unprocessed bran, 26.8 g butter (salt reduced), 0.2 g choline chloride, 4.2 g mineral mix, 1.2 g vitamin mix and 0.3 g methionine, giving 50% energy from fat, 30% energy from carbohydrate and 20% energy from protein. The quantities of vitamins and minerals added were calculated to reach the levels recommended by the American Institute of Nutrition (13). Detailed fatty acid analysis of the diet was as previously described (1), the major fatty acids being (% composition): 8:0, 1.8%; 10:0, 3.6%; 12:0, 3.6%; 14:0, 11.4%; 16:0, 28.7%; 16:1, 1.7%; 18:0, 12.3%; 18:1, 25.8%; 18:2n-6, 3.0%; 18:3n-3, 1.0%; total saturated fatty acids (FA) 61.4%; monoenoic FA, 27.5%; polyenoic FA, 4.0%. All four groups of ten rats were fed the high-fat diet for four weeks. During the final two weeks, three of the groups were supplemented orally with 90 mg/d of either LA, AA or EPA as ethyl esters. One group of rats received no PUFA supplementation and was designated as a high-fat control group.

In vitro *prostacyclin production.* At the conclusion of the four-week feeding period, the rats were anaesthetized with sodium thiopentone (75 mg/kg intraperitoneally). Blood was taken by cardiac puncture for plasma PL FA analysis. Immediately after blood collection, rats were perfused with Krebs Ringer bicarbonate buffer (KRB), pH 7.4, containing 5.5 mM glucose, to flush out the remaining blood, leaving the circulatory system free of adhering blood clots. The abdominal aorta was carefully dissected free of connective tissue and the distal 2-cm segment above the bifurcation removed. This was then split longitudinally and allowed to stabilize in KRB at 37°C for 15 min in a shaking water bath to minimize artifactual contribution of prostacyclin production due to mechanical stimulation of the arterial tissue. The artery strip was then carefully transferred to another vial and incubated in KRB for a further 15 min, after which a 200-µL aliquot was taken and stored at -20°C for RIA of 6-keto-$PGF_{1\alpha}$. At the end of the incubation, aortic segments were removed, blotted on filter paper and weighed to obtain the 'wet weight,' then freeze-dried and reweighed to obtain the 'dry weight.'

FA composition of plasma and aorta PLs. Lipid extracts were prepared from plasma and thoracic aorta by chloroform/methanol extraction (14). Following separation of the PL from the neutral lipids by TLC in the solvent system hexane/diethyl ether/acetic acid (85:15:2, by vol), the PL FA were converted to their methyl esters (15). The concentration of component FA was then determined by gas–liquid chromatography on a 50 m x 0.32 mm fused silica capillary column coated with CP-SIL 88, using diheptadecanoyl phosphatidylcholine as an internal standard, as previously described (15).

Assessment of in vivo *prostacyclin production.* Endogenous prostacyclin production (PGI_2 and PGI_3) was determined by measuring the urinary levels of the major systemic metabolite of PGI_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ and its Δ^{17} analog from PGI_3. Both were measured by stable isotope dilution assay using negative ion chemical ionization GC/MS in the selected ion monitoring mode (SIM) as has been described previously (8,16–18).

In brief, urine samples were thawed and centrifuged (2000 g for 5 min), after which a 3-mL aliquot was spiked with 10 ng tetradeuterated [19,19,20,20-$^2H_4$]2,3-dinor-6-keto-$PGF_{1\alpha}$. The endogenous compound and the internal standard were then extracted and purified by sequential steps of column chromatography and TLC. The purified compounds were then derivatized to their

methyloxime pentafluorobenzyl ester form and purified by TLC. The derivatized sample was extracted from the silica gel and further derivatized to the trimethylsilyl ether with BSTFA/pyridine. It was then dried down under nitrogen and made up in 20 µL dodecane ready for GC/MS analysis (16).

The derivatized dinor metabolite of the dienoic prostacyclin (PGI_2) and the corresponding trienoic metabolite derived from PGI_3, were quantified simultaneously by GC/MS using SIM and monitoring m/z 586 for PGI_2-M and 584 for PGI_3-M and 590 for the tetradeuterated internal standard (19). Gas chromatography was performed on a Hewlett Packard 5890, operated in splitless mode with a 12 m x 0.22 mm BP-1 fused silica capillary column. Injector temperature was 250°C, interface temperature 260°C. The oven was kept at 190°C for 1 min, then heated to 300°C at a rate of 20°C/min, then held for a further 5 min. The carrier gas was helium at a column head pressure of 87 KPa. The gas chromatograph was interfaced to a Hewlett Packard 5988A single quadrapole mass spectrometer operated in negative ion chemical ionization mode with methane as the ionization gas. Source temperature was 100°C, electron energy 70 eV, emission current 300 mA and electron multiplier voltage 1889 V.

Statistical analyses. Differences between groups were established using analysis of variance in conjunction with multiple comparison tests. Dunnet's test was used for FA analyses and *in vitro* prostacyclin. Tukey's test was used for weight gain and food consumption. *In vivo* PGI_2 and PGI_3 production were assessed using paired *t*-test. The relationship between *in vitro* and *in vivo* prostacyclin production was established using Pearson's correlation coefficient. All statistical analyses were performed on Minitab Version 8 (Minitab Inc., State College, PA). Results were expressed as mean ± SEM, $P < 0.05$ for all significant changes, unless otherwise indicated.

RESULTS

There was no significant difference in energy consumption between any of the diet groups. Similarly there was no significant difference in weight gain between the groups in the first 14 d (7.0 ± 0.3 g/d), or during the second two-week period while receiving FA supplements (3.6 ± 0.2 g/d).

Supplementation of the high-fat diet with LA resulted in a significant decline in plasma PL 22:5n-3 and 22:6n-3, with no significant change in aortic PL FA, relative to the high-fat control group (Tables 1 and 2). AA supplementation resulted in significant decreases in plasma 18:2n-6, 20:3n-9, 20:3n-6 and 22:6n-3, while 20:4n-6 showed a significant increase. In aortic PL, 18:2n-6, 20:3n-9, 20:5n-3 and 22:6n-3 all fell, while 20:4n-6 increased significantly. EPA supplementation resulted in a significant decrease in 20:4n-6, while 20:5n-3 and 22:5n-3 increased in the plasma PL. The aortic PL of this group showed a significant rise in 20:5n-3 and 22:5n-3.

Supplementation of animals on the high-fat diet with LA showed a nonsignificant trend toward increased prostacyclin production by the abdominal aorta *in vitro* (Fig. 1). The groups supplemented with AA and EPA both showed significant increases in *in vitro* prostacyclin production, with the greatest increase in the AA-

TABLE 1

Fatty Acid Composition of Plasma Phospholipids (% of total fatty acids)

Fatty acids[a]	High-fat (control) (n = 8)	High-fat + LA (n = 9)	High-fat + AA (n = 10)	High-fat + EPA (n = 10)
16:0	21.5 ± 0.6	21.7 ± 0.9	20.2 ± 0.4	21.5 ± 0.9
16:1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
18:0	19.1 ± 1.3	20.7 ± 1.4	20.0 ± 0.4	20.4 ± 0.7
18:1	11.3 ± 0.4	12.0 ± 0.6	11.6 ± 0.5	12.9 ± 0.3
18:2n-6	13.3 ± 0.6	14.2 ± 0.8	6.6 ± 0.9[b]	12.5 ± 0.7
20:3n-9	0.8 ± 0.1	0.8 ± 0.1	0.1 ± 0.1[b]	0.6 ± 0.1
20:3n-6	1.3 ± 0.1	1.6 ± 0.1	0.4 ± 0.1[b]	1.4 ± 0.1
20:4n-6	22.1 ± 0.9	20.2 ± 1.0	35.4 ± 0.9[b]	16.1 ± 0.4[b]
20:5n-3	1.2 ± 0.1	0.9 ± 0.1	0.3 ± 0.1[b]	4.4 ± 0.5[b]
22:5n-3	0.8 ± 0.1	0.6 ± 0.1[b]	0.8 ± 0.1	1.8 ± 0.1[b]
22:6n-3	8.1 ± 0.7	6.5 ± 0.3[b]	3.9 ± 0.5[b]	7.5 ± 0.8
% n-6	36.6 ± 0.9	37.3 ± 0.7	42.5 ± 0.5[b]	30.0 ± 0.6[b]
% n-3	10.2 ± 0.8	8.2 ± 0.4	5.0 ± 0.6[b]	13.3 ± 0.8[b]
n-6/n-3 ratio	3.7 ± 0.3	4.6 ± 0.2	9.2 ± 0.8[b]	2.3 ± 0.1[b]

[a]Rats were fed the high-fat diet for two weeks then supplemented with ethyl linoleate (LA), ethyl arachidonate (AA) or ethyl eicosapentaenoate (EPA) for a further two weeks.

[b]Significantly different from the high-fat control group ($P < 0.05$).

TABLE 2

Fatty Acid Composition of Aorta Phospholipids (% of total acids)

Fatty acids[a]	High-fat (control) (n = 8)	High-fat + LA (n = 8)	High-fat + AA (n = 8)	High-fat + EPA (n = 8)
14:0	1.5 ± 0.3	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
15:0	1.3 ± 0.3	1.2 ± 0.1	1.0 ± 0.2	1.1 ± 0.2
16:0	23.9 ± 0.7	23.1 ± 1.0	23.3 ± 1.1	23.3 ± 1.0
16:1	1.8 ± 0.3	1.6 ± 0.2	1.2 ± 0.1	1.4 ± 0.2
18:0	19.7 ± 1.6	21.1 ± 1.8	22.9 ± 1.5	20.9 ± 1.0
18:1	14.9 ± 0.7	16.0 ± 2.4	12.7 ± 1.5	13.9 ± 0.8
18:2n-6	6.0 ± 0.7	6.0 ± 0.4	2.9 ± 0.2[b]	5.3 ± 0.2
20:2n-6	0.8 ± 0.2	0.6 ± 0.2	0.9 ± 0.3	0.7 ± 0.2
20:3n-9	1.3 ± 0.1	1.1 ± 0.1	0.8 ± 0.1[b]	1.1 ± 0.1
20:3n-6	1.8 ± 0.1	1.6 ± 0.1	1.3 ± 0.3	1.7 ± 0.1
20:4n-6	18.5 ± 0.8	19.4 ± 1.3	22.9 ± 1.2[b]	17.5 ± 1.3
20:5n-3	1.0 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	2.8 ± 0.3[b]
22:4n-6	2.0 ± 0.2	1.6 ± 0.3	2.7 ± 0.5	1.6 ± 0.2
22:5n-6	0.9 ± 0.2	1.1 ± 0.4	1.8 ± 0.4	1.1 ± 0.3
22:5n-3	1.6 ± 0.2	1.4 ± 0.2	1.9 ± 0.1	3.1 ± 0.5[b]
22:6n-3	3.0 ± 0.1	2.4 ± 0.2	2.2 ± 0.1[b]	3.4 ± 0.3
% n-6	30.0 ± 0.9	30.2 ± 1.4	32.5 ± 1.6	27.9 ± 1.4
% n-3	5.7 ± 0.3	4.5 ± 0.4[b]	4.4 ± 0.1[b]	9.3 ± 0.6[b]
n-6/n-3 ratio	5.3 ± 0.4	6.9 ± 0.4[b]	7.4 ± 0.2[b]	3.1 ± 0.2[b]

[a]Rats were fed the high-fat diet for two weeks then supplemented with ethyl linoleate (LA), ethyl arachidonate (AA) or ethyl eicosapentaenoate (EPA) for a further two weeks.

[b]Significantly different from the high-fat control group ($P < 0.05$).

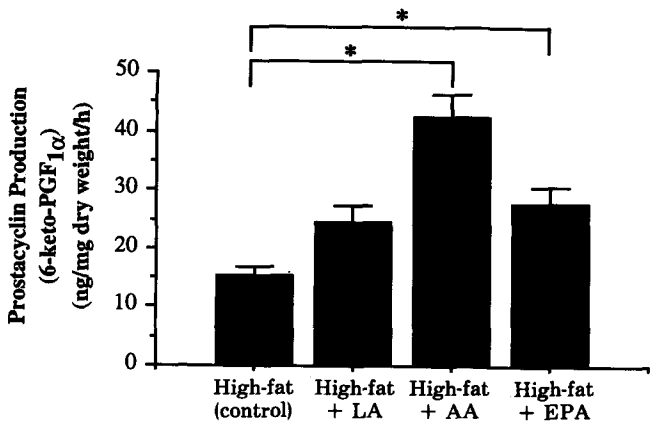

FIG. 1. *In vitro* aortic prostacyclin production in rats fed a high-fat diet and supplemented with fatty acids where indicated; measured as 6-keto-$PGF_{1\alpha}$; mean ± SEM. *Significant difference from the high-fat control diet ($P < 0.05$). Abbreviations: LA, linoleic acid (18:2n-6); AA, arachidonic acid (20:4n-6); EPA, eicosapentaenoic acid (20:5n-3).

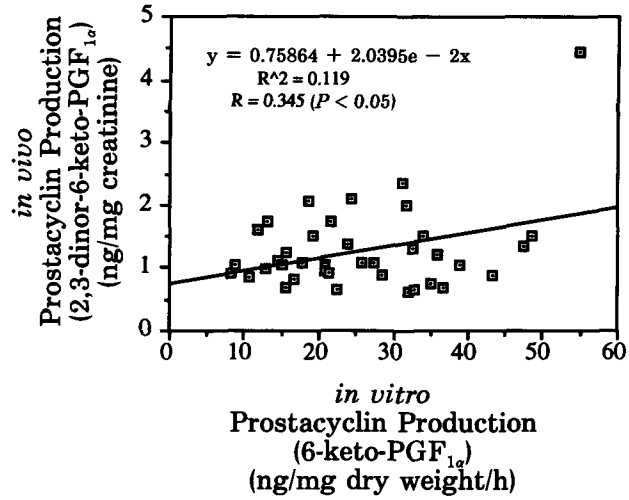

FIG. 2. Correlation of an radioimmunoassay measure of prostacyclin production *in vitro* by incubated aortic strips, with *in vivo* prostacyclin production, as measured by urinary metabolite level with gas chromatography/mass spectrometry; r = 0.345 ($P < 0.05$).

supplemented group representing a 2.8-fold increase over the high-fat control group.

Quantitative analysis was carried out for the two endogenous metabolites of prostacyclin (PGI_2-M or 2,3-dinor-6-keto-$PGF_{1\alpha}$ and PGI_3-M or Δ^{17}-2,3-dinor-6-keto-$PGF_{1\alpha}$) in each of the three dietary groups receiving FA supplements, with each group being their own control as they went from high-fat control diet to high-fat diet plus FA supplement (Table 3). AA supplementation significantly increased (66.7%, $P < 0.01$) the excretion of PGI_2-M, conversely PGI_2-M excretion was decreased significantly (60.0%, $P < 0.01$) by EPA supplementation. The PGI_3-M was significantly increased (100%, $P < 0.01$) by EPA supplementation. LA supplementation did not lead to an increase in the PGI_2-M production.

TABLE 3

The Effect of PUFA Supplementation on Production of Urinary PGI_2 and PGI_3 Metabolites as Determined by Gas Chromatography/Mass Spectrometry

Diet group[a]	PGI_2-M		PGI_3-M	
	Before	After	Before	After
High-fat (n = 10)	1.27 ± 0.13[b]		0.08 ± 0.02	
High-fat + LA (n = 10)	1.20 ± 0.12	1.13 ± 0.12	0.04 ± 0.01	0.04 ± 0.01
High-fat + AA (n = 8)	1.23 ± 0.12	2.05 ± 0.36[c]	0.05 ± 0.01	0.02 ± 0.01
High-fat + EPA (n = 9)	1.26 ± 0.15	0.80 ± 0.06[c]	0.05 ± 0.01	0.10 ± 0.01[c]

[a]Rats were fed the high-fat diet for two weeks then supplemented with ethyl linoleate (LA), ethyl arachidonate (AA) or ethyl eicosapentaenoate (EPA) for a further two weeks. Urine was collected before and after the two-week supplementation period. PUFA, polyunsaturated fatty acids; PGI, prostaglandin I.

[b]Values are means ± SEM (ng/mg creatinine).

[c]Significantly different from their own baseline ($P < 0.05$).

There was a significant positive correlation between the *in vitro* and *in vivo* methods for the determination of prostacyclin production (r = 0.345, $P < 0.05$) (Fig. 2). In addition, there was a significant positive correlation between the *in vivo* production of PGI_2-M and the AA level in aorta PL (r = 0.473, $P < 0.01$) and a significant negative correlation with the EPA level in the aorta PL (r = 0.411, $P < 0.05$).

DISCUSSION

In this study we found that dietary AA led to increases in plasma and aorta PL AA levels in the rat and that these changes were associated with an increase in both the *in vitro* and *in vivo* prostacyclin production. We have previously noted that dietary AA increases aorta PL AA levels and *in vitro* prostacyclin production; however, to our knowledge this is the first time that dietary AA has been shown to increase the production of PGI_2-M (which is considered to reflect the systemic production of prostacyclin) in any species. Falardeau and Martineau (20) found AA induced a fourfold increase in urinary PGI_2-M in rats when injected subcutaneously. These results are also in agreement with data from other groups who have shown that increased AA levels in aortic PL, achieved by manipulation of dietary LA and n-3 PUFA rather than by AA supplementation, were accompanied by increased *in vitro* prostacyclin production (21, 22).

The addition of AA to bovine arterial endothelial cells in culture leads to increases in eicosanoid production (23) presumably by increasing the substrate availability and reducing the level of competitor FA, such as EPA and DHA. This explanation may also be true in the *in vivo* situation in the rat since there was a significant positive correlation between the aorta PL AA level and the urinary PGI_2-M level and a significant negative correlation between the aorta PL EPA level and the urinary PGI_2-M level.

There was an 18% rise in the AA in the aorta PL; however, the increase in the production of prostacyclin was more than doubled in the *in vitro* assay and was increased by 67% in the *in vivo* assay. In order to account for this greater increase in prostacyclin production than the increase in the tissue AA levels, it is necessary to consider the likely pools of AA used for eicosanoid synthesis. Phosphatidylinositol and phosphatidylethanolamine are considered to be the reservoir of substrate (4) for eicosanoid synthesis, and it is possible that the AA supplementation results in a differential enrichment of AA in these particular PL. Alternatively, it has been suggested that the speed with which eicosanoids are produced from dietary AA and DHA indicates that the dietary PUFA may not have to be incorporated into tissue PL before being available as an eicosanoid precursor (24,25). Lands and others (24,26) have suggested that the likely pool is a nonesterified FA fraction.

Supplementation of the rats with the same amount of dietary LA as AA did not increase the plasma or aorta PL AA levels, nor did it alter the production of prostacyclin. It has been well established that dietary AA is a more effective precursor of tissue AA than is dietary LA in the rat (27). The reasons advanced for this difference are that AA is conserved better than LA, due to a lower rate of ß-oxidation and that LA is found in most lipid classes in tissues with only a fraction of that ingested available for conversion to AA (28). Knapp and Fitzgerald (18) have previously shown that LA supplementation (in the form of safflower oil) in humans did not increase the urinary output of 2,3 dinor-6-keto-$PGF_{1\alpha}$. This is presumably due to a failure of this dietary regime to significantly increase the tissue AA levels.

The dietary supplementation of the rats with 90 mg/d of EPA led to significant increases in the EPA level in plasma and aorta PL and to a significant reduction in AA levels in the plasma PL but not in the aorta PL. The level of urinary PGI_2-M decreased significantly following supplementation with EPA. Knapp and Salem (29) also obtained a decrease in PGI_2-M production following the dosing of rats with fish oil for 14 d, but the decrease was not significant, presumably due to the small number of animals used (n = 4). While there was no decrease in the AA level in the aorta PL in the present experiment, the decreased PGI_2-M may have been the result of a reduced availability of AA in a particular PL in the aorta, or as discussed above from the nonesterified FA pool used for eicosanoid synthesis. This result supports previous *in vitro* studies (3,30,31) and the suggestion by Needleman *et al.* (6) that EPA competitively inhibits AA metabolism by cyclooxygenase. However, it is not consistent with the results of Fischer and Weber (32), Knapp *et al.* (19) and Fischer *et al.* (25), who found no decrease in urinary PGI_2-M levels in humans fed diets rich in fish oil or purified n-3 PUFA. There is no immediate explanation for these contradictory findings; however, they may be the result of differences in eicosanoid metabolism between species, or the form in which the EPA was administered, or the dose rate. It should be noted that in the latter study of Fischer *et al.* (25) there was a substantial variation in response between individuals (2 increased, 4 decreased). The EPA supplementation led to a small but significant increase in urinary PGI_3-M, which is consistent with the data of Knapp and Salem (29) who fed fish oil (9% wt/wt) to rats for 14 d.

The *in vitro* prostacyclin production by arterial strips in the group supplemented with EPA exhibited a significantly increased prostacyclin production, although this was less marked than with AA supplementation. Other groups feeding fish oil or EPA in the free acid form have obtained significant decreases in *in vitro* prostacyclin production by rat aorta (30,33); however, Hamazaki *et al.* (34) also fed EPA to rats as the ethyl ester and found an increase in the production of prostacyclin by aortic rings using a bioassay method. The mechanism of increase in *in vitro* prostacyclin by ethyl ester of EPA has not been determined, but may be accounted for by the fact that the aortic PL 20:4n-6 level was unchanged and the 20:5n-3 was significantly increased. Thus together this could lead to an increase in total prostacyclin production, due to unaltered PGI_2 production coupled with increased PGI_3 production. It is highly unlikely that the antibody used in this RIA technique is capable of differentiating between the PGI_2-stable metabolite, 6-keto-$PGF_{1\alpha}$ and its PGI_3 analog Δ^{17}-6-keto-$PGF_{1\alpha}$. It is also possible that this is an artificial result observed after feeding the ethyl ester of EPA, but not present when fish oils or free acid form of EPA is used. We conclude that increased levels of tissue 20:5n-3 have different effects on the *in vivo* systemic production of prostacyclin and the *in vitro* production by arterial tissue in this rat model. In humans, DeCaterina *et al.* (35) found increased 6-keto-$PGF_{1\alpha}$ production *ex vivo* in arterial tissue from patients taking fish oil, following coronary bypass surgery.

The data presented here show that there is a significant positive correlation between the two methods for determination of prostacyclin, namely the *in vitro* production of 6-keto-$PGF_{1\alpha}$ by arterial preparations and the *in vivo* prostacyclin production as measured by the urinary level of one of the main prostacyclin metabolites in the rat (2,3-dinor-6-keto-$PGF_{1\alpha}$). In both methods, there is considerable variation between animals within the one dietary group. The *in vitro* method may have an inherent variability due to the procedures involved in obtaining the arterial strips from each animal and the possibility of having different proportions of endothelial cells and smooth muscle cells between preparations. In the case of the *in vivo* assay, this variation can be overcome to some extent since it is possible to measure the PGI_2-M levels before and after treatment.

Finally our results suggest that the systemic prostacyclin levels closely parallel aorta PL 20:4n-6 levels and that EPA reduces AA conversion to PGI_2 by cyclooxygenase *in vivo*, but not in aortic tissue *in vitro* in the rat. Future studies will be aimed at examining the effect of these dietary treatments on the systemic levels of thromboxane using similar GC/MS methodology. The potential physiological consequences of these dietary changes can be assessed when we have data on both these eicosanoids.

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Characterization of Calcium-Independent Cytosolic Phospholipase A_2 Activity in the Submucosal Regions of Rat Stomach and Small Intestine

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This study was undertaken to compare the calcium-independent phospholipase A_2 (PLA_2) activities in the cytosols of twelve rat tissues and to determine whether their activities were distinct. 1-*O*-Alk-1'-enyl-2-[^{14}C]-oleoyl-*sn*-glycero-3-phosphocho-line (PlsC) and 1-*O*-Alk-1'-enyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphethanolamine (PlsE) were synthesized and used as substrates, instead of phosphatidyl compounds, to exclude hydrolysis by cytosolic PLA_1 activity that could be present in some of the cytosolic preparations. For each tissue, we examined substrate specificity, pH optimum, and effect of adenosine triphosphate (ATP) and ATP analogues. PLA_2 activity was detected in eleven out of the twelve issues examined. Based on substrate specificity and pH optimum, cytosolic calcium-independent PLA_2 were classified in three groups. The first group, which included PLA_2 from small intestine, stomach and spleen, had the highest specific activity with PlsC as substrate (1253, 309 and 75 nmol/mg protein/hour, respectively) and an optimal pH at 6.5. Activity with PlsE as substrate was much lower (20–37%) than with PlsC. The second group of PLA_2 activities included the cytosolic activities from thymus, lung, liver and pancreas that showed lower specific activities for both substrates (14–23 nmol/mg protein/hour with PlsC) and had a broader optimal pH range of 6.1 to 7.5. The cytosols from brain, kidney, heart and muscle comprised the third PLA_2 group that was found to have a higher specific activity with PlsE (5–20 nmol/mg protein/hour) than PlsC and an optimal pH range from 7.4 to 7.9. Since the highest specific activity was found in the cytosol from small intestine, this PLA_2 was examined further. PLA_2 activity was found to be equally distributed in the cytosol of the submucosal portion of duodenum, jejunum and ileum with an optimal pH of 6.1 and a 5-fold higher activity with PlsC than PlsE as substrate. Moreover, this PLA_2 activity was inhibited by treatment with detergents. These results indicate the presence in the submucosal portion of the intestine of a calcium-independent cytosolic PLA_2 with a high specific activity toward PlsC and properties distinct from those described for the PLA_2 found in the intestinal brush-border.

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Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the acyl group at the *sn*-2 position of glycerophospholipids. PLA_2 plays an important role in phospholipid catabolism and in the remodeling of the acyl groups of phospholipids in general. In addition, intracellular phospholipase A_2 plays an important role in the release of arachidonic acid, which is the precursor for metabolites of the cyclooxygenase and lipoxygenase pathways (1). Although several types of PLA_2 have been purified from various tissues or biological fluids and cloned, most of them are Ca^{2+} dependent. Type I and type II secreted 14 kDa phospholipases A_2 have been extensively studied in many laboratories. Recently, cDNA encoding a calcium-dependent 85 kDa PLA_2 was cloned (2,3). In addition to these calcium-dependent PLA_2, reports in the literature are pointing to the existence of calcium-independent PLA_2 in a wide variety of animal tissues (4–20), including guinea pig intestine, hamster, dog and human hearts, bovine brain and rat parotid gland. However, a systematic evaluation of the activities between tissues has not been carried out except for the study by Pierik *et al.* (19). This has made the comparison of PLA_2 activity from various tissues difficult since the nature and presentation of the substrates, pH and composition of the buffers can influence enzymatic activity measurements.

In the present study, systematic experiments were carried out to evaluate the calcium-independent PLA_2 activity in the cytosols of twelve different rat tissues using plasmalogens as substrates. The purpose of these experiments was to identify tissues containing high calcium-independent PLA_2 specific activity and to characterize these activities in the various tissues with respect to substrate specificity, pH optimum and response to inhibitors. The data presented here also show the presence in small intestine cytosol of a calcium-independent PLA_2 with high specific activity. Since calcium-independent PLA_2 activity has been previously observed in ileal brush border (10,11), the activity detected here in the small intestine cytosol was further examined. Evidence for differences between the PLA_2 in the intestinal cytosol and that in the intestinal brush-border membrane is also provided.

MATERIALS AND METHODS

Substrates. 1-*O*-Alk-1'-enyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (plasmenylcholine, PlsC) and 1-*O*-alk-1'-enyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine, PlsE) were prepared in our laboratory according to the procedure of Paltauf and Hermetter (21). The specific radioactivity of these plasmalogens was 0.5 mCi/mmol. Plasmalogens containing 2-[^{14}C]arachidonoyl instead of 2-[^{14}C]oleoyl were also synthesized. Similarly, nonradioactive PlsC and PlsE and plasmalogens containing the 2-arachidonoyl moiety in the *sn*-2 position instead of 2-oleoyl were synthesized.

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Abbreviations: AMP-PCP, adenosine-5'(β,γ,methylene)triphosphate; AOG, 1-*O*-alk-1'enyl-2-[^{14}C]oleoyl-*sn*-glycerol; AOGP, 1-*O*-alk-1'-enyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphate; ATP, adenosine triphosphate; ATPγS, adenosine 5'-*O*-(3-thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethylannonio]-1]propanesulfonate; DTNB, 5,5'-dithio*bis*(2-nitrobenzoic aicd); HPLC, high-performance liquid chromatography; PLA_2, phopholipase A_2; PlsC, plasmenylcholine, 1-*O*-alk-1'-enyl-2-oleoyl-*sn*-glycero-3-phosphcholine; PlsE, plasmenylethanolamine, 1-*O*-alk-1'-enyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; TLC, thin-layer chromatography.

1-*O*-Alk-1'-enyl-2-[^{14}C]oleoylglycerol (AOG) and 1-*O*-alk-1'-enyl-2-[^{14}C]oleoylglycerophosphate (AOGP) were prepared from [^{14}C] PlsC by incubation with phospholipase C and phospholipase D, respectively, and subsequent purification by thin-layer chromatography (TLC).

Preparation of tissue cytosols. Male Sprague-Dawley rats (3-month-old) were subjected to ether anesthesia and killed by exsanguination. Tissues were excised and rinsed with a chilled saline solution. Each tissue was weighed, minced and homogenized on ice with a glass homogenizer or a Polytron (Brinkmann Instruments, Westbury, NY) in 2 or 3 vol of extraction buffer (0.25 M sucrose, 10 mM imidazole and 10 mM KCl at pH 7.5) containing a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin and 1 μg/mL pepstatin). Homogenates were centrifuged at 14,000 × *g* for 20 min, and the supernatants were then ultracentrifuged at 105,000 × *g* for 60 min. The final supernatant cytosolic fractions were prepared and used either immediately or after being stored at -70°C.

Preparation of mucosal and submucosal fractions from small intestine. Rat small intestine was rinsed by perfusion with cold saline containing the protease inhibitor cocktail described above. The intestine was then divided into duodenum, jejunum and ileum. Each portion was slit open on a chilled plate. Mucosa was collected by gentle scraping with a spatula and then was homogenized in 2 mL of the extraction buffer. The homogenate was ultracentrifuged at 105,000 × *g*, and the supernatant representing the mucosal cytosolic fraction was collected. The particulate fraction corresponding to the brush border fraction was treated with 1.5 mL of 1% Triton X-100 (10) for 30 min at 0°C. The mixture was centrifuged at 14,000 × *g* for 20 min, and the supernatant (solubilized brush-border membranes) was collected. After removal of mucosa, the remaining intestinal portion corresponding to the submucosa was homogenized as described above. Homogenate was centrifuged at 105,000 × *g* and the supernatant, corresponding to the submucosal cytosol, was used in the experiments.

Assay of PLA_2 activity using radiolabeled plasmalogens as substrates. PLA_2 activity was measured by the method of Dole and Meinertz (22), with slight modification, using radiolabeled PlsC and PlsE as substrates. Substrates were dispersed by sonication for 10–20 s in 0.5% Triton X-1000. The assay mixture (100 μL) consisted of a 2-fold dilution of a 100 mM wide range pH buffer (23) with 5 mM ethylenediaminetetraacetic acid, 5 mM ethyleneglycol-*bis*(β-aminoethylester)*N,N,N',N'*-tetraacetic acid, 0.125% Triton X-100, 40–45 nmol of the substrate (0.5 mCi/mmol) and the enzyme solution. Prior to performing the assay, the pH of the reaction mixture was measured in the absence of substrate. After incubation at 37°C for 7.5–60 min, liberated [^{14}C]oleic acid was isolated and measured by liquid scintillation counting. The incubation time for each cytosol was chosen so that more than 85% of the substrate remained at the end of the incubation period.

Analysis of reaction products by TLC. In some experiments, in addition to PlsC and PlsE, radiolabeled AOG and AOGP were used as substrates. At the end of the incubation period, to the reaction mixture (100 μL) was added 2 mL of chloroform/methanol (2:1, vol/vol) and 0.4 mL of water. The lower chloroform phase was collected, and the upper phase was re-extracted twice with 1 mL portions of the chloroform/methanol mixture. The combined extracts were washed twice with 1 mL of aqueous methanol (1:1, vol/vol) and dried. The residue was resolved by TLC using chloroform/methanol/ammonia (60:35:3, by vol) as solvent, and analyzed by autoradiography. Fractions were scraped from the plates and counted in a liquid scintillation counter.

Assay of PLA_2 activity by high-performance liquid chromatography (HPLC). The composition of the assay mixture was the same as that used in the standard assay except that nonradiolabeled substrate was used. At the end of the incubation at 37°C, the reaction mixture was extracted by the Dole and Meinertz procedure (22), and 1 mL of the heptane phase was dried. the residue was phenacylated (24) and analyzed by HPLC on a reverse-phase C18 column (5 m, 4.6 × 250 mm, 218TP; Vydac, The Nest Group, Southboro, MA). The reaction products were eluted isocratically with 90% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The retention times for the derivatives of arachidonic acid and oleic acid were 10 and 20 min respectively.

Protein determination. Protein concentration was measured by the Bradford method (Bio-Rad, Richmond, CA) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Presence of Ca^{2+}-independent PLA_2 in rat tissue cytosols. The Ca^{2+}-independent PLA_2 activity was examined in the cytosols of twelve tissue extracts. Activity was determined at various pH values (from pH 5 to 9) using PlsC and PlsE as substrates. PLA_2 activity was detected in each of the cytosols except in that from the submaxillary gland. The PLA_2 specific activities in the eleven tissue cytosols and their optimal pH values or optimal pH ranges are shown in Table 1. The highest PLA_2 specific activity was observed in the cytosol from small intestine, followed by stomach and spleen. Based on the pH-activity curves of PLA_2 and the specific activities measured with both PlsC and PlsE, the PLA_2 activities of the eleven cytosols can be divided into three groups. The first group included the cytosolic PLA_2 from intestine, stomach and spleen that exhibited sharp activity peak at about pH 6.5 with PlsC as substrate (Fig. 1A). Their activities towards PlsE were 64–81% lower than the ones obtained with PlsC (Fig. 1A). The second group included the cytosolic PLA_2 from lung, thymus, liver and pancreas. It also showed higher activities toward PlsC than PlsE, but the difference in activities between the two substrates was only about 2-fold. In addition, the optimal pH value for this group of cytosolic PLA_2 was about 6.5. However, the pH-activity representation for the second group showed broad peaks between 6.0 and 7.0, especially with PlsE as substrate. The third group included the cytosolic PLA_2 from brain, kidney, heart and skeletal muscle. This group was characterized by

TABLE 1

Phospholipase A_2 (PLA_2) Activity and pH Optimum in Eleven Rat Tissue Cytosols[a]

	Plasmenylcholine (PlsC)		Plasmenylethanolamine	
Tissue	pH Optimum	PLA_2 activity (nmol/mg protein/hour)	pH Optimum	PLA_2 activity (nmol/mg protein/hour)
Intestine	6.5 (6.5)	1253 ± 70	6.5 (6.5)	241 ± 8.3
Stomach	6.5 (6.5)	309 ± 40	6.5 (6.5)	79.3 ± 2.0
Spleen	6.1 (6.1)	75.4 ± 9.5	6.1 (6.1)	27.7 ± 3.9
Lung	6.2–7.5 (7.2)	23.2 ± 1.6	6.2–7.5 (7.2)	17.2 ± 0.8
Thymus	5.9–7.5 (6.3)	16.3 ± 2.6	5.9–7.5 (6.3)	6.8 ± 0.4
Liver	6.1–6.9 (6.5)	16.3 ± 0.8	6.1–7.9 (6.5)	8.3 ± 0.3
Pancreas	6.1–6.8 (6.4)	14.2 ± 1.8	6.1–7.8 (6.4)	8.5 ± 0.4
Brain	6.3–8.5 (7.9)	11.9 ± 0.8	6.3–8.5 (7.9)	19.7 ± 0.5
Kidney	6.0–8.3 (7.4)	9.6 ± 0.7	6.8–8.3 (7.4)	14.9 ± 0.4
Heart	5.5–7.8 (7.8)	5.5 ± 0.2	6.2–8.3 (7.8)	9.3 ± 0.9
Muscle	5.7–8.0 (7.5)	2.9 ± 0.1	6.6–8.5 (7.5)	4.7 ± 0.1

[a]Activity was measured by the Dole and Meinertz assay (Materials and Methods) in the presence of 1 mM adenosine triphosphate and 1 mM dithiothreitol using radiolabeled PlsC and PlsC as substrates. Measurements were done at various pH values as described in Materials and Methods and in Figure 1. Broad pH optima are shown as ranges. Values of activity are expressed in nmol/mg protein/hour and correspond to the mean ± SD from four determinations at the optimal pH indicated in parentheses.

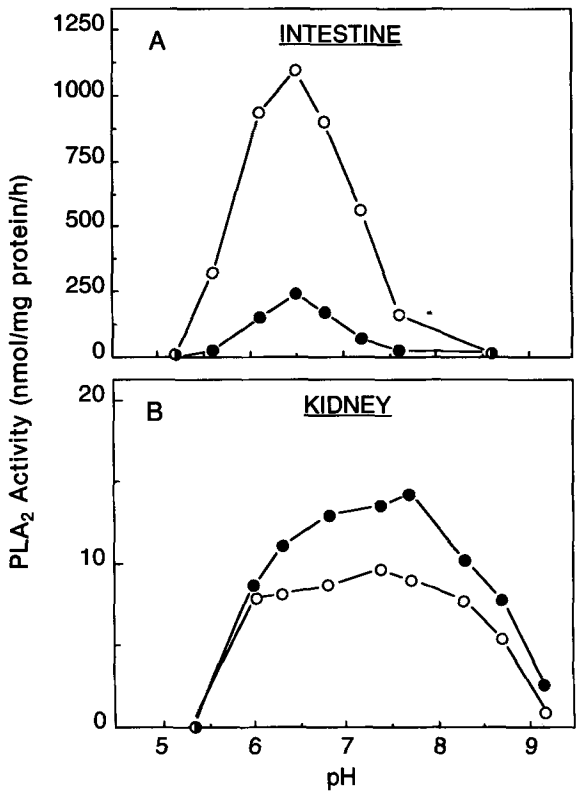

FIG. 1. The pH optimum of phospholipase A_2 activity of cytosols from intestine and kidney. Activity was measured by the Dole assay (Materials and Methods) using plasmenylcholine (PlsC) (○) and plasmenylethanolamine (PlsE) (●) as substrates. Each reaction mixture contained 1 mM adenosine triphosphate and 1 mM dithiothreitol. A. Cytosol of small intestine, 80 μg of protein was incubated for 7.5 min with PlsC and 15 min with PlsE. B. Cytosol of kidney, 525 μg of protein ws incubated for 60 min with either one of the substrates. Each point is the mean of duplicate analysis.

higher activities toward PlsE than PlsC (about 2-fold) at essentially all pH tested. As illustrated for the kidney cytosol (Fig. 1B), the pH-activity curves for this group showed broad peaks between pH 6 and 8.5. The Ca^{2+}-independent PLA_2 activities in the cytosols from rat kidney, heart, pancreas, spleen liver, lung and brain have been detected previously (19). However, the specific enzymatic activities we found in our assays were from 4-fold higher for brain to 96-fold higher for spleen than those reported previously (19). These differences could be attributed to the differences in assay conditions, such as pH, buffer composition, preparation and the nature of substrates. In addition, the data in Table 1 show the presence of PLA_2 activities in the cytosols from small intestine, stomach, thymus and skeletal muscle that had not been described previously.

The PLA_2 activities given above were obtained by measuring the release of radiolabeled oleic acid from the *sn*-2 position of the substrates. Since the assay was done with cytosolic extracts, additional experiments were carried out to determine whether the release of radiolabeled oleic acid resulted from the direct action of PLA_2 only or whether other enzymes present in the cytosols indirectly contributed to this activity. We therefore used two other substrates, namely AOG and AOGP, in addition to PlsC and PlsE. At the end of the incubation time, the reaction mixtures were extracted and analyzed by TLC. The results obtained with the cytosols from intestine and kidney are shown in Figure 2. The TLC analysis of reaction mixture obtained by incubating intestinal cytosol (80 μg protein) with PlsC (45 nmol) is shown in lane 1 of Figure 2A. In this case, about 25% of the radioactivity of the substrate was recovered as oleic acid (11 nmol), and little radioactivity was recovered from fractions corresponding to AOG or AOGP. When AOG (12 nmol) or AOGP (8 nmol) were incubated with the same amount of intestinal cytosol, little release of

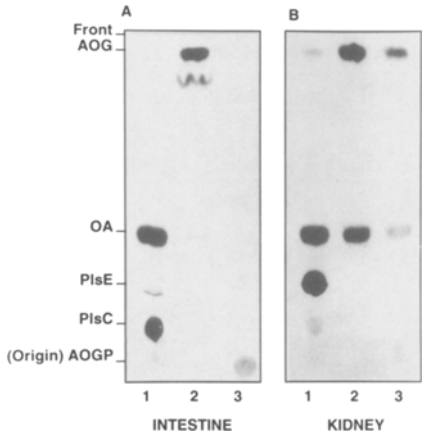

FIG. 2. Thin-layer chromatography (TLC) analysis of the reaction products obtained by incubating tissue cytosols with different radiolabeled substrates. A. 80 μg of intestinal cytosol extract was incubated for 7.5 min at 37°C with either 45 nmol of [^{14}C]PlsC (lane 1), 12 nmol of [^{14}C]1-*O*-alk-1'-enyl-2-[^{14}C]oleyl-*sn*-glycerol (AOG) (lane 2) or 8 nmol of [^{14}C]1-*O*-alk-1'-enyl-2-[^{14}C]oleyl-*sn*-glycero-3-phosphate (AOGP) (lane 3). B. 525 μg of kidney cytosolic extract was incubated for 60 min at 37°C with either 45 nmol of [14C]PlsE (lane 1), 12 nmol of [14C]AOG (lane 2) or 8 nmol of [14C]AOGP (lane 3). At the end of the incubation period, reaction products were extracted and separated by TLC on a silica gel plate as described in Materials and Methods. Products were analyzed by autoradiography on the plate. Fractions were also scraped from the plates and counted in a scintillation counter. The minor band in lane 1 at the position of PlsE (panel A) corresponds to a contamination. The identity of the fraction below AOG in lane 2 (panel A) is unknown; OA, oleic acid. See Figure 1 for other abbreviations.

radiolabeled oleic acid was observed (lanes 2 and 3 of Fig. 2A). In the case of kidney cytosol (525 μg protein), 8 nmol of oleic acid was produced from PlsE (45 nmol), 4 nmol from AOG (12 nmol) and 1 nmol from AOGP (8 nmol), respectively (lanes 1–3, Fig. 2B). In contrast to intestinal cytosol, kidney cytosol released relatively large amounts of oleic acid from AOG and converted most of AOGP to AOG. However, in both cases, little accumulation of AOG and AOGP was observed when PlsC or PlsE was used as substrate. Radioactivity was only recovered in the AOG or AOGP fraction when AOG and AOGP themselves were used as substrates, indicating that no hydrolysis took place under these conditions. The results suggest that the release of oleic acid in the presence of the cytosolic extract from intestine or kidney did not take place *via* formation and subsequent hydrolysis of AOG and AOGP but rather directly from the plasmalogens. Similar results were obtained from these analyses that were carried out with the cytosolic extracts from brain, stomach, spleen and liver (data not shown). Thus, the radiochemical method used in this work appears appropriate for measuring PLA_2 activity in rat tissue cytosols.

*Effects of ATP and 5,5'Dithio*bis*(2-nitrobenzoic acid) (DTNB).* In a preliminary investigation we found that ATP and dithiothreitol increased PLA_2 activity of kidney cytosol. Therefore, we examined the effects of ATP, adenosine 5'-*O*-(3-thio)triphosphate (ATPγS) and DTNB on PLA_2 activity of eleven cytosols. All cytosolic fractions examined lost 10 to 40% of their PLA_2 activities in the absence of ATP when PlsE served as substrate

TABLE 2

Effects of ATP and ATPγS on Phospholipase A_2 Activity of Tissue Cytosols[a]

	Plasmenylcholine		Plasmenylethanolamine	
Tissue	–ATP	–ATP + ATPγS[b]	–ATP	–ATP + ATPγS[b]
Intestine	99 ± 0.2	99 ± 0.1	67 ± 2.5	123 ± 2.3
Stomach	86 ± 2.4	93 ± 0.3	73 ± 0.1	104 ± 1.4
Spleen	81 ± 1.2	88 ± 1.7	75 ± 1.3	107 ± 0.9
Lung	95 ± 3.2	_[c]	88 ± 1.2	99 ± 0.5
Thymus	72 ± 5.7	_	61 ± 1.3	97 ± 0.3
Liver	83 ± 0.1	_	60 ± 0.5	94 ± 1.0
Pancreas	91 ± 0.7	_	78 ± 3.3	93 ± 0.5
Brain	98 ± 0.3	_	78 ± 1.9	93 ± 3.2
	88 ± 1.7	_	63 ± 2.4	100 ± 1.2
Heart	100 ± 4.2	_	81 ± 2.9	101 ± 1.2
Muscle	82 ± 2.2	_	90 ± 0.1	106 ± 0.1

[a]Activity was measured by the Dole and Meinertz assay (Ref. 22); the complete reaction system contained 1 mM adenosine triphosphate (ATP) and 1 mM dithiothreitol in the standard mixture (Materials and Methods). Assays for each cytosol were carried out at the optimal pH used for activity measurement as in Table 1. Each data point is the mean ± SD of duplicate analyses and expressed as percent of the activity of each complete system. Specific activity of the complete system of each cytosol was essentially the same as that given in Table 1.

[b]Adenosine 5'-*O*-(3-thio)triphosphate (ATPγS) was added at 1 mM.

[c]Not measured.

TABLE 3

Substrate Specificity of PLA_2 Activity in Various Cytosols[a]

	Plasmenylcholine (nmol/mg protein/hour)		Plasmenylethanolamine (nmol/mg protein/hour)	
	2-Oleoyl	2-Arachidonoyl	2-Oleoyl	2-Arachidonoyl
Intestine	1545 ± 49	969.0 ± 15	310.0 ± 5.0	206.0 ± 8.0
Stomach	338.0 ± 20	282.0 ± 24	72.0 ± 1.0	52.0 ± 4.0
Spleen	87.0 ± 5.0	74.0 ± 5.0	39.0 ± 0.3	24.0 ± 0.6
Brain	15.0 ± 0.8	4.2 ± 0.1	22.0 ± 2.5	6.6 ± 0.6
Kidney	8.3 ± 0.8	5.4 ± 0.1	17.0 ± 5.3	9.0 ± 0.9
Muscle	3.2 ± 0.8	1.3 ± 0.07	5.8 ± 0.07	1.5 ± 0.07

[a]Phospholipase A_2 (PLA_2) activity was measured with unlabeled substrates. At the end of the incubation period, reaction products were extracted and analyzed by high-performance liquid chromatography as described in Materials and Methods. Each data point is the mean ± SD of duplicate analyses and expressed as nmol/mg protein/hour.

(Table 2). Activity was mostly restored to the level observed with ATP by addition of ATPγS. In the case of intestinal cytosol, ATPγS gave a much higher PLA_2 activity than ATP. When PlsC was used as substrate, omission of ATP resulted in a smaller decrease than when PlsE was used. It is not clear whether or not these results point toward the presence of two types of PLA_2. Similar or slightly lower activities were observed in the presence of adenosine-5'(β,γ-methylene)triphosphate (AMP-PCP) instead of ATP or ATPγS. The fact that AMP-PCP is as efficient as ATP and ATPγS in activating or stabilizing PLA_2 in the presence of high concentrations of chelators would indicate that the phosphorylation or hydrolysis of ATP is not involved in the increase of PLA_2 activity with ATP.

According to Hazen *et al.* (14,15), Ca^{2+}-independent PLA_2 of myocardial cytosol is stimulated and stabilized by ATP, AMP-PNP and ATPγS. As similar stimulation or stabilization effect of ATP on PLA_2 activity was observed in rat parotid gland (18) and in an adipogenic cell line (Fukushima and Serrero, submitted for publication). Our present results suggest that modulation of Ca^{2+}-independent PLA_2 by ATP is not specific for cardiac PLA_2 and also occurs in other tissues (Table 2).

The effect of DTNB on PLA_2 activity was also examined. DTNB (0.1 mM) inhibited by 78 to 98% cytosolic PLA_2 activity in ten of eleven tissues studied. Liver cytosolic PLA_2 activity was not inhibited by 0.1 mM DTNB, but complete inhibition was observed with 1 mM DTNB. In our studies, rat lung cytosolic PLA_2 was completely inhibited by DTNB. However, a DTNB-insensitive PLA_2 has also been purified from rat lung cytosol (6). These data suggest that similar to Ca^{2+}-independent PLA_2 of the myocardial cytosol (15), PLA_2 in most rat tissue cytosols assayed requires the presence of intact thiol(s) for activity.

Substrate specificity of PLA_2 activity. PLA_2 activities from six tissues that represented each of the three PLA_2 groups previously defined were measured by HPLC using 2-oleoyl and 2-arachidonoyl PlsC and PlsE as substrates (Table 3). None of the cytosolic extracts examined showed higher activities with 2-arachidonoyl species of either of the plasmalogens compared to the 2-oleoyl species. By contrast, it has been previously reported that calcium-independent PLA_2 from myocardium, which is stabilized by ATP, showed a 2-fold higher activity for plasmalogens containing arachidonoyl instead of oleoyl in the 2-position (15).

Further examination of PLA_2 activity in rat small intestine and stomach. A Ca^{2+}-independent PLA_2 activity had previously been detected and partially characterized in the brush border of rat and guinea pig ileum (10,11). This PLA_2 activity had an alkaline pH optimum, was localized in the brush border, was solubilized with endogenous proteinases (10) and was activated by bile salts and by 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (10,11). Based on these characteristics, this PLA_2 activity was assumed to play a role in lipid digestion. Therefore, the PLA_2 activity that we detected in the small intestine was further characterized, and its properties were compared to those of ileal brush border PLA_2. Rat small intestine was divided into the duodenum, jejunum and ileum, and PLA_2 activity was measured in the cytosols of the submucosa and the mucosa and also in the solubilized brush-border membranes of each portion. In all samples, activity was measured with PlsC and PlsE as substrates, at pH 6.5 or pH 8.5, and in the absence or in the presence of CHAPS. As illustrated in Table 4, the submucosal cytosols from the three small intestine regions showed high PLA_2 activities when assayed with PlsC at pH 6.5, indicating this PLA_2 activity is not localized in one particular region of the small intestine. In the three cases, only 15–25% of the activity was observed with PlsE as substrate when compared with PlsC. Very little activity was detected at pH 8.5 with either of the substrates. The activity of the cytosols was fully inhibited with 1% CHAPS. Deoxycholate (0.2%) also inhibited the activity of the cytosols (data not shown). The mucosa cytosols from the duodenum and jejunum showed activity patterns similar to those of the submucosal cytosols although the specific activities were 60% lower. However, the cytosol of ileal mucosa was found to be different. In the absence of CHAPS, PLA_2 activity measured with PlsC as substrate was high at pH 6.5 and very low at pH 8.5; with PlsE, low activity was observed at both pH. In

TABLE 4

PLA_2 Activity in Rat Small Intestine[a]

	PLA_2 activity (nmol/mg protein/hour)							
	Plasmenylcholine (PlsC)				Plasmenylethanolamine (PlsE)			
	pH 6.5		pH 8.5		pH 6.5		pH 8.5	
CHAPS (1%)	–	+	–	+	–	+	–	+
Submucosa cytosol								
Duodenum	818	7	24	10	168	7	21	5
Jejunum	735	12	24	21	126	10	20	14
Ileum	1268	49	26	80	189	59	17	91
Mucosa cytosol								
Duodenum	261	16	32	13	78	16	31	15
Jejunum	224	15	14	17	40	15	13	20
Ileum	580	267	46	343	73	285	29	447
Solubilized mucosa membranes								
Duodenum	43	27	34	26	20	16	23	17
Jejunum	24	32	17	37	15	34	15	38
Ileum	111	682	87	837	50	753	52	854

[a]Rat small intestine was divided into duodenum, jejunum and ileum. Mucosa was separated from submucosa, and brush-border membranes were prepared according to the procedure described in Materials and Methods. Phospholipase A_2 (PLA_2) activity was measured with 1 mM adenosine and 1 mM dithiothreitol using PlsC and PlsE at pH 6.5 and 8.5 without (–) or with (+) 1% 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Values are expressed as nmol/mg protein/hour.

the presence of CHAPS, activity toward PlsC was decreased by more than 50% at pH 6.5 but was increased more than 7-fold at pH 8.5. With PlsE as substrate, activity increased in the presence of CHAPS and was similar to that obtained with PlsC at both pH. The solubilized brush-border membrane fraction of the ileum showed high activity with both substrates only in the presence of CHAPS, and the activity was slightly higher at pH 8.5 than 6.5. In this case, little difference in activities was seen with PlsE and PlsC as substrates. The results shown in Table 4 indicate that the calcium-independent cytosolic PLA_2 described here is preferentially distributed in the submucosa of all three portions of the intestine. In contrast, CHAPS-activated PLA_2 is localized mainly in the ileal brush-border membranes and appears to be similar to the PLA_2 already reported by Pind and Kuksis (10).

In summary, submucosal cytosolic PLA_2 has a very narrow pH optimum at pH 6.5, is inhibited by CHAPS and deoxycholate and its specific activity toward PlsC is much higher than that toward PlsE. By contrast, brush border PLA_2 has a broad pH optimum, requires CHAPS for full activity and shows little substrate selectivity between PlsC and PlsE. This indicates that the PLA_2 activity found in cytosol of submucosa of the small intestine has properties that are distinct from those of the brush-border PLA_2 described previously.

Since high PLA_2 activity was also detected in the stomach (Table 1), its distribution was examined. Cytosol prepared from the submucosa of the stomach showed a slightly higher activity than cytosol from the mucosa (330 *vs.* 230 nmol/mg protein/hour). As observed with PLA_2 from small intestine, PLA_2 activity from stomach was fully inhibited with 0.2% deoxycholate (data not shown).

The data presented here demonstrate the presence of calcium-independent PLA_2 activities in eleven of the twelve rat tissues cytosols examined suggesting that cytosolic calcium-independent PLA_2 is quite ubiquitous. Based on specific activity, substrate specificity and pH optimum, we divided these PLA_2 into three classes, each with different properties. At this point our data do not allow us to conclude that this classification reflects the existence of various isoforms of calcium-independent PLA_2 in different tissues, as this diversity could equally reflect the heterogeneous nature of the membrane lipid constituents in the different tissues serving as natural PLA_2 substrates. A more detailed characterization of the PLA_2 activities in the different tissues will be necessary to determine whether these activities correspond to different molecular species or PLA_2 isoforms or are due to the variation in sample composition. Recently, the existence of two isoforms of calcium-independent PLA_2 has been reported in human myocardium (25). One form with a pH optimum of 7.0 is the major form and has a preference for arachidonoyl plasmalogens, whereas the other form, which is a minor form, has an optimum pH of 8.5 and hydrolyzes other phospholipids as well as plasmalogens. In addition, two distinct types of calcium-independent PLA_2 have been characterized from bovine brain cytosol (26).

The data reported here are also of interest because they demonstrated the presence of a calcium-independent PLA_2 with a high specific activity in mucosal and submucosal fractions of small intestine, which has different properties from the brush border PLA_2 character-

ized previously (10,11). The role of this cytosolic PLA_2 is not clear. Various prostaglandins are synthesized in the stomach and intestine, and it is known that these organs are highly sensitive to prostaglandins (27,28). To date, the release of arachidonic acid for prostaglandin synthesis has mainly been attributed to calcium-dependent cytosolic PLA_2 activity (2). However, a recent report showed that calcium-independent PLA_2 mediated the hormonally-induced release of arachidonic acid in aortic smooth muscle cells (29). PLA_2 activities so far characterized in the gastrointestinal tract include group I PLA_2 in the gastric mucosa, group II PLA_2 and calcium-independent PLA_2 present in secretory glands and in brush-border membranes (30–32). Based on their location, all these PLA_2 activities appear to function extracellularly. Yet, the cytosolic intestinal PLA_2 described here appears to be different. Although this PLA_2 is not arachidonic acid-specific, its role could be to supply fatty acid, including arachidonic acid within tissues. Since the PLA_2 activity in the intestine was very high, this PLA_2 may play an important role in intracellular lipid metabolism in this tissue. Moreover, our data indicate that submucosal portions of the intestine appear to be adequate sources for the further characterization of this PLA_2.

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The Reaction of Low Levels of Nitrogen Dioxide with Methyl Linoleate in the Presence and Absence of Oxygen

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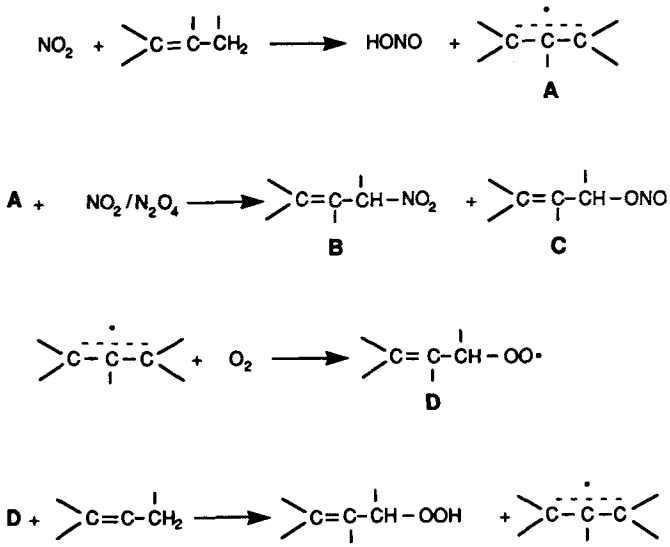
The reaction of methyl linoleate with low levels of nitrogen dioxide in a carrier gas, such as helium or air, at nitrogen dioxide concentrations ranging from 2 to 228 ppm was studied and the products formed were monitored. In both aerobic and anaerobic conditions, low concentrations of nitrogen dioxide reacted with methyl linoleate predominately to form allylic products. When a 1:1 mixture of methyl palmitate/methyl linoleate was layered over an aqueous buffer and a nitrogen dioxide stream was passed from underneath, so that the stream passed through the aqueous layer before contacting the organic layer, allylic products again predominated. In the absence of air, the allylic products consisted of allylic nitro and nitrite derivatives of linoleate, whereas in the presence of air, allylic hydroperoxides were the principal products. The findings suggest that fatty acids with doubly allylic hydrogen atoms react preferentially by a hydrogen atom abstraction reaction rather than by the addition of nitrogen dioxide to a double bond.
***Lipids* 29, 171–176 (1994).**

Nitrogen dioxide has been shown to initiate the autoxidation of unsaturated fatty acids both *in vivo* and *in vitro* (1–6). The oxidation of lung lipids is thought to be involved in the pathology caused by the inhalation of nitrogen dioxide-containing polluted air (1–6), which includes diseases such as pulmonary edema, bronchitis, pulmonary fibrosis and perhaps cancer (7–9). We have previously shown that low concentrations of nitrogen dioxide react with cyclohexene predominantly by a hydrogen atom abstraction mechanism rather than by the addition mechanism that had been assumed to apply (10,11). We also have identified the allylic nitrate and nitro compounds formed when methyl linoleate and methyl linolenate react with NO_2 in the absence of oxygen (12). Recently, Postlethwait and Bidani (13) have reported that ^{15}N-labeled NO_2 was not taken up by the lipids of isolated, perfused rat lungs that were exposed to NO_2; they interpreted these results to mean that nitrogen dioxide reacts *in vivo* by a H atom abstraction mechanism.

According to our H atom abstraction mechanism (10,11), nitrogen dioxide abstracts a hydrogen atom from an allylic or doubly allylic position, forming a resonance-stabilized radical and nitrous acid (see Scheme 1). In the absence of oxygen, the allylic radical **(A)** can combine with a molecule of nitrogen dioxide to form an allylic nitro **(B)** or nitrite compound **(C)**. In the presence of oxygen, the radical reacts to form a peroxyl radical **(D)**.

Nitrogen dioxide can also react with alkenes by an additional mechanism. According to this mechanism (10,11), nitrogen dioxide adds to the double bond in a reversible step (14), forming a carbon-centered radical (compound **E** in Scheme 2). In the absence of oxygen, the carbon-centered radical can react with a second molecule of nitrogen dioxide, forming a dinitro **(F)** or nitro–nitrite compound **(G)**. These dinitro or nitro–nitrite compounds can eliminate nitrous acid to form a vinyl nitro or vinyl nitrite compound **(H)**. In the presence of oxygen, the carbon-centered radical can react with a molecule of oxygen to form a nitro-peroxyl radical **(I)** (15).

In the present paper, we report studies of the reaction of nitrogen dioxide (2–230 ppm in helium or air) with neat methyl linoleate and a solution of methyl



SCHEME 1

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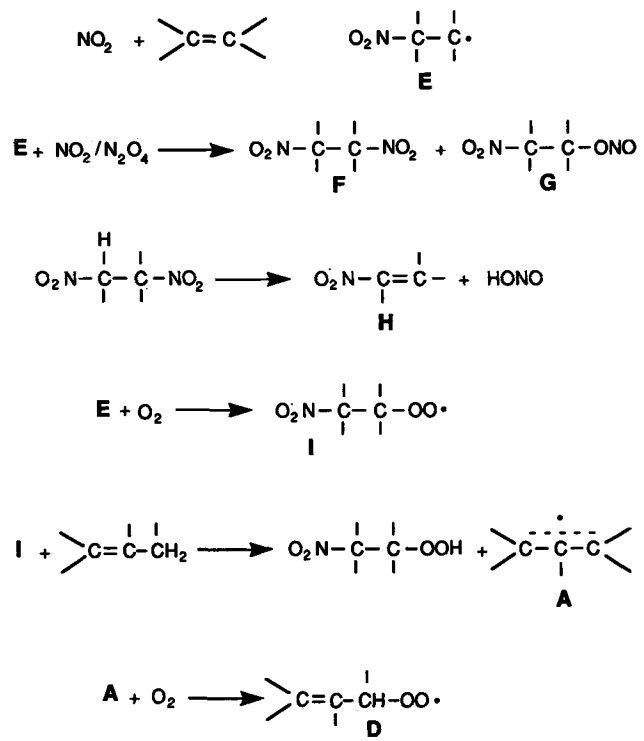
Abbreviations: AN, allylic nitro or nitrite compounds; *c*, *cis* double bond; DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; NCI, negative chemical ionization; NMR, nuclear magnetic resonance; *t*, *trans* double bond; UV, ultraviolet; vinyl nitro, mixture of methyl 9-nitro-9,12-octadecadienoate and methyl 13-nitro-9,12-octadecadienoate; vinyl nitrate, mixture of methyl 9-nitrato-9,12-octadecadienoate, methyl 10-nitrato-9,12-octadecadienoate, methyl 12-nitrato-9,12-octadecadienoate, and methyl 13-nitrato-9,12-octadecadienoate; *c,t*-18:2, mixture of methyl *cis*-9,*trans*-12-octadecadienoate and methyl *trans*-9,*cis*-12-octadecadienoate; 9*c*,11*t*-13AN, mixture of methyl 13-nitrito-*cis*-9,*trans*-11-octadecadienoate and methyl 13-nitro-*cis*-9,*trans*-11-octadecadienoate, 9*c*,11*t*-13HP, methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate; 9*t*,11*t*-13AN, mixture of methyl 13-nitrito-*trans*-9,*trans*-11-octadecadienoate and methyl 13-nitro-*trans*-9,*trans*-11-octadecadienoate; 9*t*,11*t*-13HP, methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate; 10*t*,12*c*-9AN, mixture of methyl 9-nitrito-*trans*-10,*cis*-12-octadecadienoate and methyl 9-nitro-*trans*-10,*cis*-12-octadecadienoate; 10*t*,12*c*-9HP, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate; 10*t*,12*t*-9AN, mixture of methyl 9-nitrito-*trans*-10,*trans*-12-octadecadienoate and methyl 9-nitro-*trans*-10,*trans*-12-octadecadienoate; 10*t*,12*t*-9HP, methyl-9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate.



SCHEME 2

linoleate/methyl palmitate (1:1 molar) layered over a sodium phosphate buffer.

EXPERIMENTAL PROCEDURES

Materials. Methyl linoleate [99% pure by gas chromatography (GC) Sigma, St. Louis, MO] was purified from hydroperoxide contaminants by passing the ester in oxygen-free hexane [high-performance liquid chromatography (HPLC) grade, Mallinckrodt, St. Louis, MO] sequentially through four Pasteur pipette columns containing 1.0 g of alumina (neutral; Aldrich, Milwaukee, WI). The last column contained 0.02 g of diethylenetriaminepentaacetic acid (DTPA) in the tip to remove any trace metals. The columns were rinsed with oxygen-free hexane before adding the unsaturated fatty acid ester solution. The entire purification process was performed in a glove bag under an atmosphere of nitrogen. The hexane was evaporated using a stream of nitrogen.

Methyl palmitate, methyl elaidate, methyl linolelaidate (*trans*-9, *trans*-12-octa-decadienoate; 99% by GC; Sigma) and *d*-carvone (96% pure; Aldrich) were used as internal standards for GC and HPLC analysis. Dinitrogen tetroxide (99.5% pure; Matheson, Secaucus, NJ) was added to a glass bulb containing phosphorus pentoxide (97% pure; Aldrich) and purged of oxygen with argon by freezing and thawing three times. Hexane, isopropanol (HPLC grade; Mallinckrodt), and helium (99.9999% chromatographic grade; Air Products, Allentown, PA) were also used. A thymol trap was made by dissolving 0.4 g of thymol (Sigma) and 4 g of sodium hydroxide (97% pure; EM Science, Gibbstown, NJ) in 1 L of deionized water.

Instrumentation. Ultraviolet (UV) spectra were taken on a Hewlett-Packard (Avondale, PA) 8451 A diode array spectrophotometer; infrared (IR) spectra were obtained on an IBM (Armonk, NY) IR/45 spectrometer. HPLC on a Varian (Palo Alto, CA) 5000 series instrument with a UV detector set at a 215 nm wavelength with a 16 nm slit width was used to analyze the reaction mixtures. A 25 × 0.46 cm silica column (Rainin, Woburn, MA) was used with hexane/isopropanol (99:1, vol/vol, oxygen free) as solvent system at a flow rate of 1 mL/min. Proton magnetic resonance [^{1}H nuclear magnetic resonance (NMR)] analyses were performed on a Bruker (Billerica, MA) 200 MHz instrument using deuterated chloroform as solvent. A Hewlett-Packard 5890 GC/mass spectrometer equipped with a 100 m × 0.25 mm SP-2560 fused silica column was used to separate the *cis/trans* isomers of methyl linoleate. The column was maintained isothermally at 230°C for 15 min, and the mass range 10–500 amu was scanned. A gas chromatograph (Varian 3740 series) equipped with a flame-ionization detector and a 100 m × 0.25 mm SP-2560 fused silica column maintained isothermally at 210 and 200°C was used to determine the amount of addition/elimination products formed in the reactions. The GC injection port was maintained at 250°C. Negative chemical ionization (NCI) (0.5 Torr methane) was performed on a Finnigan MAT (San Jose, CA) TSQ 4500 mass spectrometer using a direct exposure probe ramped to 1 A at a rate of 10 mA/s and scanning a mass range of 44–800 amu. The source temperature was 150°C and the manifold temperature was 100°C.

The reaction of methyl linoleate with nitrogen dioxide in a carrier gas. The bubbler apparatus shown in Figure 1 was assembled in a glove bag under an atmosphere of nitrogen. The nitrogen dioxide bulb was opened slightly and the nitrogen dioxide gas flow equilibrated for one hour. The concentration of nitrogen dioxide in a helium

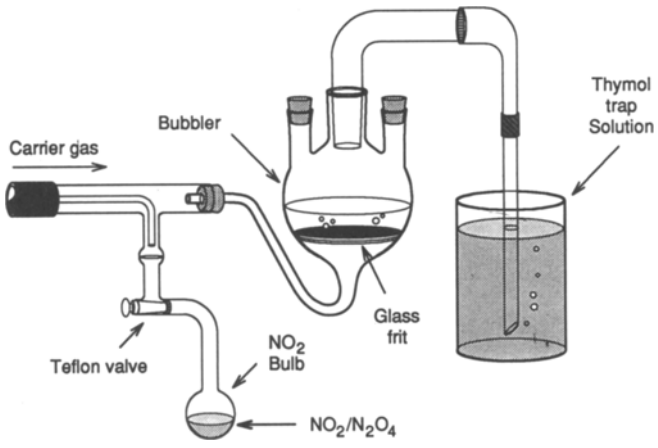


FIG. 1. The apparatus used for the reactions. A low level of NO_2/N_2O_4 is placed in the bulb to allow a low rate of delivery of NO_2 to the system. The NO_2 bulb is opened slightly and equilibrated for one hour. A one-hour blank is run to determine NO_2 concentration in the carrier gas. The methyl linoleate is placed on the glass frit, and NO_2 in the carrier gas is bubbled through the fatty acid ester for one hour.

or air carrier gas at flow rates of 60 and 30 mL/min were determined by bubbling the nitrogen dioxide into a 1 L or 100 mL thymol trap for 1 h at 37°C. In the anaerobic reactions, ultrapure helium was the carrier gas and in the aerobic reactions compressed air was used. The efficiency of the thymol trap shown in Figure 1 was 70 ± 4.0 and 83 ± 2.1% for the 100 mL and 1 L trap solutions, respectively. All runs were one hour long.

The difference in the amount of nitrogen dioxide in the thymol trap solution used with the blank vs. that used with the reaction solution was taken as the amount of nitrogen dioxide incorporated into the unsaturated fatty acid esters. The amount of nitrogen dioxide in the thymol traps was determined using the Saltzman analysis (16). For some of the lower concentrations of nitrogen dioxide, the Saltzman analysis was not sensitive enough to measure the small amounts of unreacted nitrogen dioxide; for these runs, the total amount of nitrogen dioxide delivered was used.

For studies using fatty acids layered over an aqueous layer, nitrogen dioxide in a carrier gas was bubbled through the buffer to determine the amount of nitrogen dioxide/nitrogen tetroxide that was consumed by a dismutation reaction to form nitrate and nitrite ions.

The reaction of nitrite/nitrate anions with methyl linoleate at 37°C in the absence of oxygen. A solution that was 1.1×10^{-3} M in both sodium nitrite and sodium nitrate in a sodium phosphate buffer (pH 7.4; 0.5 M) containing DTPA was prepared. This concentration of nitrite and nitrate anions is equivalent to the concentration of these ions that would be formed from the disproportionation of 228 ppm of NO_2 in air at a flow rate of 60 mL/min bubbling through 10 mL of buffer for one hour (17). The nitrite/nitrate solution was purged of oxygen and allowed to react with methyl linoleate for one hour.

The study of fatty acids over an aqueous layer. Nitrogen dioxide introduced into the bottom of the apparatus was bubbled through a buffer (approximately 4 cm in height) consisting of 10 mL of 0.5 M sodium phosphate (pH 7.4) before coming in contact with a methyl palmitate/methyl linoleate (1:1 molar) solution (approximately 0.4 cm in height) layered on the surface of the buffer. The organic layer of the aqueous reaction mixture was extracted with dichloromethane, dried with $MgSO_4$, filtered and concentrated by blowing a stream of nitrogen over the solution.

Identification of products. A mixture of methyl *cis*-9,*trans*-12-octadecadienoate and methyl *trans*-9,*cis*-12-octadecadienoate isomers (*c,t*-18:2) was analyzed by GC/MS (18). The isomers, which have identical electron ionization (EI) mass spectra to those of methyl linoleate and methyl linolelaidate (18), were identified by their order of elution. The same elution order of the geometrical isomers of methyl linoleate under similar GC conditions was reported by Kobayashi (19).

Methyl 9-nitro-9,12-octadecadienoate, and methyl 13-nitro-9,12-octadecadienoate (vinyl nitro isomers) coeluted in HPLC and GC analysis. The mixture of isomers was isolated by HPLC from a methyl linoleate and nitrogen dioxide reaction mixture and was identified by NMR, IR and NCI. Proton NMR showed signals at 5.4 ppm (*br, m,* 3H vinyl), 3.66 ppm (*s,* 3H CH_3O), 2.7 ppm (*dd,* 2H), and 2.3 ppm (*t,* 2H). The vinylic proton absorption appearing at 5.4 ppm instead of about 7 ppm is caused by the substitution of a nitro and an alkyl group on the same vinylic carbon. This type of upfield shift has previously been seen with other electron-withdrawing functional groups, e.g., crotonic acid vs. methacrylic acid and 1-bromo-1-propene vs. 2-bromopropene (20). IR: nitro 1552 cm^{-1}, 1362 cm^{-1}, *cis* double bond 723 cm^{-1}, and a vinylic double bond 989 cm^{-1} (21). Methane NCI was used to determine the molecular weight *m/z* 339 and to confirm the presence of a nitro functional group by the appearance of a *m/z* 46 ion (22). NCI $[M^-] = m/z$ 339 (9%), $[M - H]^- = m/z$ 338 (30%), $[C_8H_{13}O_2N]^- = m/z$ 155 (<1%), $[C_7H_{13}]^- = m/z$ 97 (<1%), $NO_2^- = m/z$ 46 (100%).

Methyl 9-nitrato-9,12-octadecadienoate, methyl 10-nitrato-9,12-octadecadienoate, methyl 12-nitrato-9,12-octadecadienoate, methyl 13-nitrato-9,12-octadecadienoate (vinyl nitrate) also coeluted in HPLC and GC. The mixture of isomers was isolated by HPLC and identified by NMR, IR and NCI analysis. NMR: 5.40 ppm (*br, m,* 3H vinyl), 3.66 ppm (*s,* 3H CH_3O), 2.7 ppm (*dd,* 2H) and 2.3 ppm (*t,* 2H). The IR spectrum of the mixture of isomers has absorbances at 1634 cm^{-1} (NO_2 asymmetrical stretch), 1257 cm^{-1} (symmetrical vibration), 856 cm^{-1} (N-O stretch) and 682 cm^{-1} (NO_2 vibration); these absorbances demonstrate the presence of a nitrate functional group (20). The NCI analysis was used to determine the molecular weight and to confirm the presence of a nitrate functional group by the appearance of an *m/z* 62 ion (21). NCI: $[M^-] = m/z$ 355 (15%), $[M - H]^- = m/z$ 354 (9%), $[M - O]^- = m/z$ 339 (12%), $[M - OH]^- = m/z$ 338 (8%), $[M - H_2O]^- = m/z$ 337 (28%), $NO_3^- = m/z$ 62 (37%), and $NO_2^- = m/z$ 46 (100%). The hydroperoxide and allylic nitro (nitrite) isomers of methyl linoleate were identified by NMR and NCI (12).

Methyl 9-nitrato-*trans*-10,*trans*-12-octadecadienoate, which elutes after the hydroperoxides of methyl linoleate, was isolated and identified by NCI analysis. The position of the functional group was obtained from the detection of an *m/z* 151 ion in the NCI spectrum, which is indicative of the fragmentation of the compound in the source to form 2,4-decadienal. NCI: $[M^-] = m/z$ 355 (17%), $[M - NO_2]^- = m/z$ 309 (25%), $[M - H_2NO_2]^- = m/z$ 307 (39%), $[M - CH_3NO_3]^{-1} = m/z$ 277 (52%), $[M - C_9H_{18}NO_4]^{-1} = m/z$ 151 (28%), $NO_3^{-1} = m/z$ 62 (100%), $NO_2^{-1} = m/z$ 46 (65%).

RESULTS

Reaction of nitrogen dioxide with methyl linoleate. We have measured the yields of products from the reaction of nitrogen dioxide with neat methyl linoleate as well as with an equimolar solution of methyl palmitate/methyl linoleate layered over an aqueous buffer. The product compositions formed using either helium or air as the carrier gas are presented later in Tables 1–4. The data shown are averages from five HPLC analyses and four GC analyses of the reaction mixture using *d*-carvone

TABLE 1

Moles of Products Formed from the Reactions of NO_2 and Methyl Linoleate Under Helium at 37°C[a]

	ppm NO_2		
	6.8	30	81
% Conversion 18:2	5.1	7.4	10
Addition products $\times 10^{-7}$ moles			
c,t-18:2	1.4 ± 0.1	2.4 ± 0.2	5.0 ± 0.5
Vinyl nitro	0.0	0.0	36 ± 0.9
Total moles of addition products $\times 10^{-7}$	1.4 ± 0.1	2.4 ± 0.2	41 ± 1.0
Allylic products $\times 10^{-7}$ moles			
9*c*,11*t*-13AN	7.7 ± 0.1	8.5 ± 0.1	9.3 ± 0.5
9*t*,11*t*-13AN	8.8 ± 0.5	11 ± 1	11 ± 0.3
10*t*,12*c*-9AN	7.2 ± 0	8.0 ± 0.2	8.5 ± 0.5
10*t*,12*t*-9AN	8.8 ± 0.5	10 ± 0.5	11 ± 0.5
Total moles of H abstraction products $\times 10^{-7}$	32 ± 0.7	38 ± 1.0	40 ± 0.9
Moles products $\times 10^{-7}$	34 ± 0.7	40 ± 1.0	81 ± 1.3
Moles NO_2 reacted $\times 10^{-7}$	9.6 ± 0.5	42 ± 2.4	110 ± 6.3
KCL[b]	3.5 ± 0.2	0.95 ± 0.06	0.74 ± 0.04

[a]For clarity, standard errors (determined from at least five analyses) are shown only in this table; however, the data presented in all of the tables have similar errors (see Ref. 16).

[b]KCL (kinetic chain length) is defined as the moles of products formed divided by the moles of nitrogen dioxide reacted. *c,t, cis, trans; c,t*-18:2, mixture of methyl *cis*-9, *trans*-12-octadecadienoate and methyl *trans*-9, *cis*-12-octadecadienoate; 9c, 11*t*-13AN, mixture of methyl 13-nitrito-*cis*-9, *trans*-11-octadecadienoate and methyl 13-nitro-*cis*-9, *trans*-11-octadecadienoate; 9*t*,11*t*-13AN, mixture of methyl 13-nitrito-*trans*-9,*trans*-11-octadecadienoate and methyl 13-nitro-*trans*-9,*trans*-11-octadecadienoate; 10*t*,12*c*-9AN, mixture of methyl 9-nitrito-*trans*-10,*cis*-12 octadecadienoate and methyl 9-nitro-*trans*-10,*cis*-12-octadecadienoate; 10*t*,12*t*-9AN, mixture of methyl 9-nitrito-*trans*-10,*trans*-12-octadecadienoate and methyl 9-nitro-*trans*-10,*trans*-12-octadecadienoate.

and methyl linolelaidate as internal standards (17). The response factor for the hydroperoxides was used to quantify the allylic nitrite(nitro) compounds as the nitrite(nitro) compounds decompose when the solvent is removed. The concentration of nitrogen dioxide in the carrier gas, the identity of the carrier gas (He or air) and the percent conversion is given in each table. As before (10), we have used the nature of the products, allylic or nonallylic, to assess the fraction of nitrogen dioxide that reacted by a hydrogen atom abstraction or addition mechanism. Products that are assigned to arise from an addition mechanism (*c,t*-18:2, vinyl nitro and vinyl nitrate) and an H atom abstraction mechanism (9*c*,11*t*-13AN, 9*c*,11*t*-13HP, 9*t*,11*t*-13AN, 9*t*,11*t*-13HP, 10*t*,12*c*-9AN, 10*t*,12*c*-9HP, 10*t*,12*t*-9AN, and 10*t*,12*t*-9HP) are tabulated separately in each table. The total moles of products detected and nitrogen dioxide that reacted are listed at the bottom of the tables. The values for the kinetic chainlength (KCL), defined as the moles of products formed divided by the moles of nitrogen dioxide reacted, also are given. The products identified account for 96 ± 12 mole % of the methyl linoleate that was consumed in the reactions.

Anaerobic reactions of methyl linoleate. Table 1 displays the identity and quantity of products formed when neat methyl linoleate is allowed to react with various concentrations of nitrogen dioxide in ultrapure helium. In agreement with our previous results (10,11), these low concentrations of nitrogen dioxide react predominantly with methyl linoleate by an H atom abstraction mechanism.

Aqueous reactions of methyl linoleate. To determine whether low concentrations of nitrogen dioxide can pass through an aqueous environment and abstract a hydrogen atom from methyl linoleate, nitrogen dioxide was bubbled through a buffer with a methyl palmitate/methyl linoleate (1:1, molar) solution layered on top of the surface. Table 2 displays the moles of products formed in this system.

In this system, the *c,t*-18:2 addition/elimination product is not detected at the low concentrations of nitrogen dioxide used. Allylic nitrite isomers are presumed to be formed but to be hydrolyzed to allylic alcohols (23). Therefore, the H abstraction products are taken to be the sum of the allylic nitro and allylic alcohol isomers, compounds that co-elute under our HPLC conditions. An equimolar mixture of nitrate and nitrite anions did not react with methyl linoleate under our experimental conditions (17).

TABLE 2

Moles of Products Formed in the Reactions of Nitrogen Dioxide with 50:50 Mixtures of Methyl Palmitate/Methyl Linoleate in the Aqueous System Under Helium at 37°C

	ppm NO_2			
	4.3	19.7	140	215
% Conversion 18:2	1.60	1.90	2.80	5.90
Addition products $\times 10^{-7}$ moles				
c,t-18:2	0.0	0.0	0.79	1.7
Vinyl nitro	0.0	0.0	0.0	19
Total moles of addition products $\times 10^{-7}$	0.0	0.0	0.79	21
Allylic products $\times 10^{-7}$ moles				
9*c*,11*t*-13AN	1.3	1.8	1.7	2.9
9*t*,11*t*-13AN	2.7	3.5	4.0	6.9
10*t*,12*c*-9AN	1.1	1.6	1.6	2.6
10*t*,12*t*-9AN	2.7	3.7	4.3	6.9
Total moles of H abstraction products $\times 10^{-7}$	7.8	11	12	19
Moles products $\times 10^{-7}$	7.8	11	12	40
Moles NO_2 reacted $\times 10^{-7}$	6.0	14	16	46
KCL[a]	1.3	0.79	0.75	0.87
% NO_2 in buffer	0.0	16	41	56

[a]KCL (kinetic chain length) is defined as the moles of products formed divided by the moles of nitrogen dioxide reacted. *c,t, cis, trans;* 9c, 11*t*-13AN, mixture of methyl 13-nitrito-*cis*-9, *trans*-11-octadecadienoate and methyl 13-nitro-*cis*-9, *trans*-11-octadecadienoate; 9*t*,11*t*-13AN, mixture of methyl 13-nitrito-*trans*-9,*trans*-11-octadecadienoate and methyl 13-nitro-*trans*-9,*trans*-11-octadecadienoate; 9AN, mixture of methyl 9-nitrito-*trans*-10,*cis*-12 octadecadienoate and methyl 9-nitro-*trans*-10,*cis*-12-octadecadienoate; 10*t*,12*t*-9AN, mixture of methyl 9-nitrito-*trans*-10,*trans*-12-octadecadienoate and methyl 9-nitro-*trans*-10,*trans*-12-octadecadienoate.

TABLE 3

Moles of Products Formed from the Reactions of NO_2 and Methyl Linoleate Under Air at 37°C

	ppm NO_2			
	2.4	15	30	179
% Conversion 18:2	32	41	44	63
H abstraction products $\times 10^{-7}$ moles				
9*c*,11*t*-13HP	49	67	77	170
9*t*,11*t*-13HP	62	85	98	210
10*t*,12*c*-9HP	47	65	74	170
10*t*,12*t*-9HP	62	85	98	200
10*t*,12*t*-9AN[a]	1.3	1.3	1.3	7.1
Total moles of H abstraction products $\times 10^{-7}$	220	300	350	760
Total moles products $\times 10^{-7}$	220	300	350	760
Moles NO_2 reacted $\times 10^{-7}$	3	18	15	170
KCL[b]	73	17	23	4.5

[a]Allylic nitrates of methyl linoleate.

[b]KCL is defined as the moles of products formed divided by the moles of nitrogen dioxide reacted. Abbreviations as in Table 1; 9*c*,11*t*-13HP, methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate; 9*t*,11*t*-13HP, methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate; 10*t*,12*c*-9HP, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate; 10*t*,12*t*-9HP, methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate.

Aerobic reactions of nitrogen dioxide with methyl linoleate. Reactions of nitrogen dioxide with methyl linoleate also were performed in air (Tables 3 and 4). When air was used as the carrier gas, only hydrogen atom abstraction products were detected. The *c,t*-18:2 addition/elimination product and the nitro-hydroperoxide isomers of methyl linoleate were not detected; the only nitrogen-containing compound detected was the allylic isomer, methyl-9-nitrato-*trans*-10,*trans*-12-octadecadienoate.

TABLE 4

Moles of Products Formed from the Reactions of NO_2 with a 50:50 Mixture of Methyl Palmitate/Methyl Linoleate in the Aqueous System Under Air at 37°C

	ppm NO_2			
	4.3	29	43	228
% Conversion 18:2	7.9	34	34	47
H abstraction products $\times 10^{-7}$ moles				
9*c*,11*t*-13HP	5.0	27	18	55
9*t*,11*t*-13HP	11	57	39	110
10*t*,12*c*-9HP	4.7	25	17	52
10*t*,12*t*-9HP	12	57	39	110
10*t*,12*t*-9AN[a]	0.0	0.0	0.0	2.1
Total moles of H abstraction products $\times 10^{-7}$	33	166	113	329
Total moles products $\times 10^{-7}$	33	166	113	329
Moles NO_2 reacted $\times 10^{-7}$	2	21	18	78
KCL[b]	17	7.9	6.3	4.2
% NO_2 in buffer	0.0	26	9.5	67

[a]Allylic nitrates of methyl linoleate.

[b]KCL is defined as the moles of products formed divided by the moles of nitrogen dioxide reacted. Abbreviations as in Tables 1 and 3.

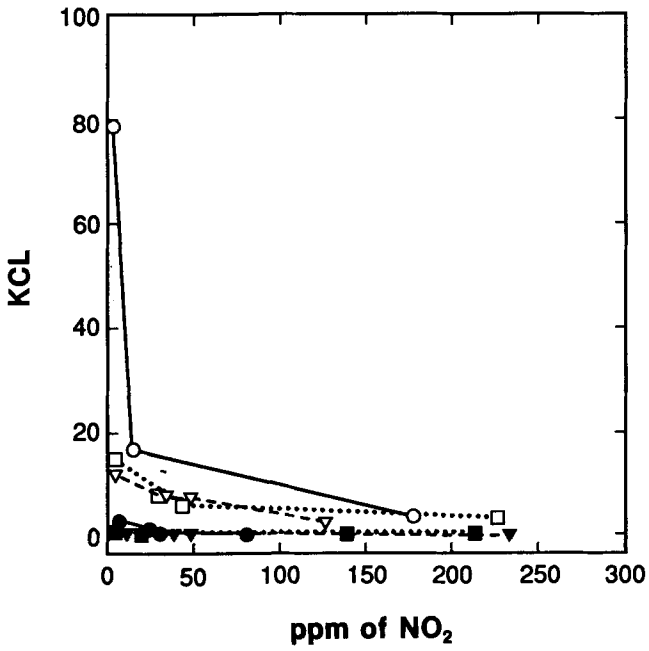


FIG. 2. The kinetic chain length (KCL) (moles of products/moles of NO_2 reacted) for the reactions of nitrogen dioxide and methyl linoleate in the absence (closed symbols) and the presence (open symbols) of oxygen. Open circle, neat methyl linoleate in air; closed circle, neat methyl linoleate in the absence of air; open triangle, a 50:50 mixture of methyl linoleate and methyl palmitate in air (Ref. 16); closed triangle, the same mixture in the absence of air (Ref. 16); open square, a 50:50 mixture of methyl linoleate and methyl palmitate in the aqueous system under air; closed square, the same mixture in the aqueous system without air.

KCL. KCL values, calculated from the moles of products formed per mole of nitrogen dioxide reacted, are shown in Figure 2 for the anaerobic and aerobic reactions of nitrogen dioxide with methyl linoleate. The KCL is larger in the aerobic reactions than in the anaerobic reactions. Diluting methyl linoleate with methyl palmitate decreases the KCL of the reactions (Fig. 2).

DISCUSSION

Methyl linoleate H abstraction and addition products. Our studies demonstrate that under anaerobic conditions, low concentrations of nitrogen dioxide predominantly abstract a hydrogen atom from methyl linoleate (Tables 1 and 2; Scheme 1). Small yields of products formed *via* the addition mechanism (Scheme 2), which predominates at high concentrations of nitrogen dioxide, were detected at low concentrations of nitrogen dioxide under anaerobic conditions (Tables 1 and 2). In agreement with our earlier results (10,11), in the absence of oxygen and as the concentration of nitrogen dioxide decreased, an increasing fraction of the reaction occurred by the H atom abstraction mechanism.

KCL. The KCL is approximately unity for most of the reactions run in the absence of air (Tables 1 and 2). However, for some of the anaerobic reactions, the chain

length is somewhat larger than one (Tables 1 and 2). A KCL greater than one in anaerobic reactions can be explained by the decomposition of the allylic nitrite compounds to an alkoxyl radical and nitric oxide (24). [Isolated allylic nitrite(nitro) isomers of the unsaturated fatty acid esters decompose when concentrated.] The alkoxyl radical formed in this decomposition could abstract a hydrogen atom from methyl linoleate, giving a carbon-centered radical. Reaction of the carbon-centered radical with nitric oxide will result in the formation of a nitroso compound that can rearrange to an oxime (21); the presence of an oxime was supported by the appearance of an IR absorption at 1587 cm^{-1} (25). This observation is in agreement with our earlier study of the reaction of nitrogen dioxide with cyclohexene in the absence of oxygen, in which the amount of oxime formed increased as the concentration of nitrogen dioxide decreased (10).

Aqueous reactions. Nitrogen dioxide was shown to be able to diffuse through an aqueous environment and then to react with unsaturated fatty acids. Bubbling nitrogen dioxide through a buffer solution over which a solution of methyl palmitate/methyl linoleate (1:1, molar) had been layered gave products that were primarily allylic, indicating that methyl linoleate reacted primarily by a hydrogen atom abstraction mechanism in this experimental design (Table 2).

Explanation for addition products not being detected in aerobic reactions. Table 4 shows a similar reaction system in which air was used as the carrier gas. Again allylic products were formed, but in this case they were primarily the allylic hydroperoxides, resulting from the chain autoxidation of methyl linoleate. In the presence of oxygen, the carbon-centered radical formed by the addition of nitrogen dioxide to the double bond of an unsaturated fatty acid can be trapped by oxygen (Scheme 2) (10,11). Accordingly, the addition/elimination product of methyl linoleate (*c,t*-18:2) was not detected in the presence of oxygen (Tables 3 and 4). The nitro-hydroperoxides that could be formed in addition reactions also were not detected, possibly because of the limit of detection of the HPLC UV detection method (Tables 3 and 4). The only nitrogen-containing products detected in aerobic reactions at low concentrations of NO_2 were allylic nitrates; these may have originated from the decomposition of peroxynitrates (which may have been formed from nitrogen dioxide reacting with peroxyl radicals) (26–28). Peroxyl radicals, of course, would result from the autoxidation of methyl linoleate, a chain reaction that would result in larger yields of peroxyl radicals than of individual addition or H atom abstraction products.

Rationale for the change in the mechanism from addition to H atom abstraction. The H atom abstraction mechanism (Scheme 1) predominates over the addition mechanism (Scheme 2) at low concentrations of nitrogen dioxide because the addition mechanism is reversible (10,11). Thus, the irreversible H atom abstraction step predominates at low NO_2 concentrations.

In conclusion, our results demonstrate that low concentrations of nitrogen dioxide in the absence of oxygen react predominantly with methyl linoleate by an H atom abstraction mechanism in either the presence or the absence of water. We propose that the same mechanism occurs in the presence of oxygen. These results corroborate the findings of Postlethwait and Bidani (13), as well as our earlier results (10,11) that *in vivo*, environmental concentrations of nitrogen dioxide will react with polyunsaturated fatty acids in pulmonary lipids exclusively by an H atom abstraction mechanism, a process that can initiate autoxidation.

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Vitamin E Isomers in Grain Amaranths (*Amaranthus* spp.)

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Vitamin E isomers are important antioxidants, but their variation is poorly documented in pseudocereal grains such as amaranths. Using normal-phase, high-performance liquid chromatography with fluorescence detection, seeds of thirteen amaranth (*Amaranthus cruentus* L., *A. hypochondriacus* L.) accessions were surveyed for the composition of tocols. The most common tocols found were α-tocopherol (2.97 to 15.65 mg/kg seed) and β-tocotrienol (5.92 to 11.47 mg/kg seed) and γ-tocotrienol (0.95 to 8.69 mg/kg seed), while some *A. cruentus* accessions contained δ-tocotrienol (0.01 to 0.42 mg/kg seed). This is the first report of tocotrienols in amaranths. *Amaranthus cruentus* grain-types of Mesoamerican origin had significantly ($P \leq 0.01$) greater levels of four tocols than did *A. cruentus* African vegetable-types. Unlike many cereal grains, amaranths have significant amounts of both β- and γ-tocotrienols; however, β-tocopherol was not detected in any of the amaranths. Using multiple linear regressions, α-tocopherol variation of both species and types was consistently explained by variation in tocols other than α-tocopherol. On the whole, fresh amaranth samples of both species tended to have higher levels of tocotrienols than samples stored for two years. Storage effects on amaranth tocol composition are suspected.

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Tocotrienols (T3) are unsaturated analogs of vitamin E, which have been recently shown to regulate cholesterol metabolism (1,2), to have antitumor activity (3) and to possess high *in vitro* antioxidative activity (4). Both tocopherols (T) and T3 have important antioxidant properties and are widespread in cereals (5). However, limited data on tocol (T and T3) variation exist for grain germplasm, for breeding materials and for different varieties.

Among the pseudocereals, grain amaranths, including *Amaranthus caudatus* L., *A. cruentus* L. (CRU) and *A. hypochondriacus* L. (HYP), have gained attention due to the high quality protein content of the seed (6). In some parts of the world, such as Africa, grain amaranths like CRU are used predominantly as vegetables rather than as grains and are termed vegetable-type amaranths. Similarly, grain-type amaranths are cultivars with a high harvest index (ratio of grain to total phytomass).

Grain amaranth germplasm contains 5–9% lipids, with most lipids concentrated in the seed coat-germ fraction (7). Becker (8) has reviewed the nutritional implications of amaranth seed oil. As far as we are aware, published data on amaranth tocols include brief reference to total seed tocopherols for three grain *Amaranthus* species (69.4 to 82.8 mg % g [sic] of total T), including *A. caudatus*, CRU and *A. mantegazzianus* Passerini (9), to CRU T (10), and to leaf T in *A. retroflexus* L. (11). Therefore, a more complete characterization of *Amaranthus* tocols is needed. The objective of this study was to evaluate the tocol composition of amaranth accessions, including domestic grain-type cultivars and germplasm representing diverse origins and types, grown over several environments.

MATERIALS AND METHODS

Seed origin and experimental design. Thirteen amaranth accessions of HYP or CRU were surveyed using seed from three field experiments (Table 1). Experiment 1 included accessions 9–12 grown in three replicate, randomized blocks at Rosemont (MN) Experiment Station, University of Minnesota in 1989. Experiment 2 included HYP grain cultivars, var. K343 and K432, grown in three replicate, randomized blocks at Grand Rapids, Albert Lea and Morris (MN) in 1989. Experiment 3 included accessions 1 through 8 grown in two replicate, randomized blocks at the North Central Regional Plant Introduction Station (Ames, IA) in 1987. Experiment 3 seed was stored for two years at 4°C and 40% relative humidity, while seed from Experiments 1 and 2 was tested immediately after the growing season.

TABLE 1

Names, Species, Types and Origins for Amaranth Accessions

Accession	Species[a]	Type[b]	Origin
1. Ames 1959	CRU	African vegetable	Ghana
2. Ames 1968	CRU	African vegetable	Ghana
3. Ames 1973	CRU	African vegetable	Nigeria
4. Ames 2000	CRU	African vegetable	Benin
5. Ames 2041	CRU	Mexican grain	Indonesia
6. PI 433228	CRU	Guatemalan grain	Guatemala
7. PI 451711	CRU	Guatemalan grain	Guatemala
8. PI 477913	CRU	Mexican grain	Mexico
9. A 200 D[c]	CRU	Mexican grain	Illinois
10. Mt-3[c]	CRU	Mexican grain	Montana
11. PI 477914	CRU	Mexican grain	Mexico
12. K343[c]	HYP	Nepal grain	Pennsylvania
13. K432[c]	HYP	Nepal grain	Pennsylvania

[a]CRU, *Amaranthus cruentus* L.; HYP, *A. hypochondriacus*.

[b]Types follow Kauffman and Reider (28) except for their African grain-types, which are, in fact, vegetable landraces and have been renamed as African vegetable-types.

[c]Accessions registered as cultivars or unregistered varieties in commerce. Mt-3 is the parent material of the cultivar "AMONT" (29) and K343 is the cultivar, "Plainsman" (30).

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Abbreviations: CRU, *Amaranthus cruentus* L.; HPLC, high-performance liquid chromatography; HYP, *A. hypochondriacus* L.; LSD, least significant difference; T, tocopherol(s) (α-T, β-T, γ-T, δ-T); T3, tocotrienol(s) (α-T3, β-T3, γ-T3, δ-T3).

Tocol analyses. Amaranth seeds were ground with a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO) into a fine powder (1 g) and extracted with 7.0 mL methanol in a disposable screw-capped tube in a variable speed shaker for 20 min, and centrifuged at 2000 rpm for 10 min. The supernatant was then transferred into a second disposable conical tube, and solvent was removed under vacuum at 40°C using an BI Vortex evaporator (Haake Buchler Instruments, Inc., Saddle Brook, NJ). Tocols (T and T3) were extracted with hexane (2 mL) by shaking on a horizontal shaker for 2 min, and the liquid was transferred into a 0.3 mL injection vial. The vial was capped and centrifuged in an Eppendorff 5413 centrifuge (WACO, Chicago, IL) for 4 min. The material was injected into the high-performance liquid chromatography (HPLC) unit for separation and quantitation of various tocols and standards according to the method of Hakkarainen *et al.* (12).

The HPLC system that was used consisted of a Model 6000A HPLC pump (Waters, Milford, MA), a Model RF-535 fluorescence monitor (Schimadzu, Wood Dale, IL) set at an excitation wavelength of 295 nm and an emission wavelength of 330 nm, a Model 231/401 auto-sampler injector (Gilson, Middleton, WI) equipped with a 20-microliter loop and a Waters 10-micrometer silica column, 30 cm × 4.0 mm i.d. Chromatograms were recorded and peak areas determined by using a Schimadzu Model C-R3A integrator. The mobile phase was 0.2% (vol/vol) 2-isopropanol/hexane at a flow rate of 1.0 mL/min. The injection volume was 30 microliters. All solvents used were chromatographic grade. Pure standards of the eight common vitamers of E were extracted from palm or barley oil and were purified by silicic acid column chromatography (1,2). Peaks from amaranth samples were identified by comparing their scan spectra (Fig. 1) with spectra of pure standards.

Statistical analyses. Data from all experiments were analyzed by one-way or two-way analysis of variance and by multiple linear regression (13). For Experiment 2, a combined analysis of variance was performed on each tocol. Homogeneity of error variances was tested before combining sites (14). For Experiment 3, orthogonal contrasts were computed using four African vegetable-types and four grain-types of Mesoamerican origin. When the F test indicated a significant effect, the differences between the means were analyzed using a protected least significant difference (LSD) test. Pooled phenotypic correlations, similar to those used when evaluating indirect selection criteria in amaranth germplasm (15), were assessed within species and types.

RESULTS AND DISCUSSION

Evaluation of tocol composition. Seven of the eight reported tocols, excluding β-T, were present in the amaranths surveyed (Tables 2–4). The γ-T, α-T3 and δ-T3 were sometimes absent from, or not detected in, the accessions. For Experiment 1, HYP var. K343, contained significantly ($P \leq 0.05$) higher levels of both α-T and γ-T3 than did the three CRU grain-type accessions (Table 2). Using two commercial HYP cultivars in Experiment 2, data on tocol stability over environments was mostly inconclusive due to heterogeneous error variances among sites (Table 3). The only significant ($P \leq 0.05$) difference between locations was that of α-T at the Grand Rapids and Morris sites. In Experiment 3, orthogonal contrasts indicated that grain-types of Mesoamerican origin contained significantly ($P \leq 0.01$) greater concentrations of γ-T, α-T3, γ-T3 and δ-T3 in the seed than did African vegetable-types (Table 4). Similar orthogonal contrasts of

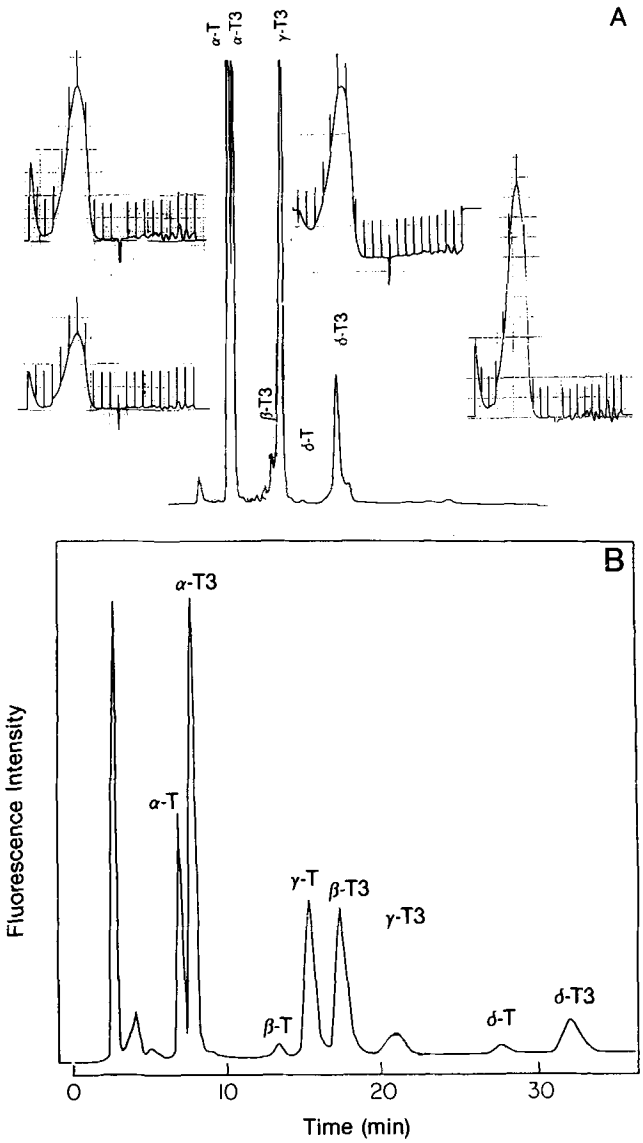

FIG. 1. Separation of tocols by high-performance liquid chromatography with fluorescence detection. The scan from the amaranth sample (A) is compared with and quantitated against a sample containing purified standards of the four tocopherols and four tocotrienols (B). The amaranth sample (A) was scanned from 240 to 440 nm for each peak, with the λ maximum for each tocol component occurring from 290 to 300 nm. The eluting solvent was 0.5% isopropanol in hexane and the chart speed was 2 mm/min. The eight pure tocol standards (B) were run with 0.2% isopropanol in hexane as solvent and at a chart speed of 5 mm/min (12). T3, tocotrienol; T, tocopherol.

TABLE 2

Vitamin E Isomers Contained in Amaranth Seed Grown at Rosemont (MN), 1989 (Experiment 1)

Acession	Tocopherols α	β	γ	δ	Tocotrienols α	β	γ	δ	Total tocols	Tocotrienols as percent of total tocols
					(mg/kg seed)					
Mt-3	4.46	0	0.02	0	0.35	10.33	1.25	0.23	16.64	73
A 200 D	3.94	0	0	0.01	0.07	8.97	1.28	0.08	14.35	73
K343	8.91	0	0.05	0.01	0	9.57	8.69	0	27.23	67
PI 477914	3.17	0	0	0	0.11	7.97	0.95	0.16	12.36	74
LSD (0.05)[a]	2.08	NS[a]	NS	NS	0.23	NS	1.50	0.10	5.42	

[a]NS, not significant; LSD, least significant difference.

TABLE 3

Effect of Location on Vitamin E Isomers in Amaranth Cultivars Grown in Minnesota During 1989 (Experiment 2)[a]

Location	Tocopherols α[b] K343	α K432	β K343	β K432	γ K343	γ K432	δ K343	δ K432	Tocotrienols α K343	α K432	β K343	β K432	γ K343	γ K432	δ K343	δ K432
								(mg/kg seed)								
Grand Rapids	5.14	2.93	0	0	0	0	0	0	0	0.01	7.24	6.97	8.31	7.96	0	0
Albert Lea	9.45	7.34	0	0	0.26	0.01	0.01	0.02	0.01	0	9.60	11.44	7.10	6.06	0	0.03
Morris	8.37	7.93	0	0	0.07	0.05	0	0.01	0	0	9.06	11.47	8.22	7.58	0	0

[a]Except for α-tocopherol, all locations were not significantly different for tocols or the error variances at sites were found heterogeneous by Barlett's test; thus, it was inappropriate to combine some sites for analysis of variance.

[b]Highly significant effects ($P \leq 0.01$) between Morris and Grand Rapids were found.

total tocols and of T3 as a percent of tocols were not significant, while the latter measure had a $P \leq 0.07$.

The data obtained represent the first report of T3 in grain amaranths, with β-T3 and γ-T3 being most prevalent and ranging from 5.92 to 11.47 mg/kg seed and from 0.95 to 8.69 mg/kg seed, respectively. The magnitude and range of these values are typical of cereal grains (16). The δ-T3 isomers were rarely detected in HYP grains, but they ranged from none to 0.42 mg/kg seed in CRU grains. Comparatively, CRU has δ-T3 concentrations akin to those in rice (*Oryza sativa* L., 0.2 mg/kg seed), but less than the δ-T3 levels in two-row barley (*Hordeum distichon* L., 0.6 mg/kg seed) and bulrush millet (*Pennisetum americanum* L., K. Leeke, 2.0 mg/kg seed) (16). Among the tocopherols, α-T was most abundant in the tested amaranths, ranging from 2.97 to

TABLE 4

Vitamin E Isomer Content of Amaranth Germplasm Grown at Ames (IA) in 1987 (Experiment 3)

Acession	Tocopherols α	β	γ	δ	Tocotrienols α	β	γ	δ	Total tocols	Tocotrienols as percent of total tocols
	(mg/kg seed)									
African vegetable-types										
Ames 1959	12.44	0	0.01	0.02	0.01	8.05	1.64	0	22.17	44
Ames 1968	11.49	0	0.02	0.01	0.01	7.52	1.34	0.01	20.40	44
Ames 1973	11.54	0	0	0.02	0.01	8.16	1.52	0	21.25	45
Ames 2000	10.77	0	0.01	0.02	0	5.92	1.06	0	17.78	39
Mesoamerican grain-types										
Ames 2041	9.43	0	0.05	0	0.09	7.92	2.25	0.19	19.93	52
PI 433228	12.13	0	0	0	0.01	7.16	1.67	0.13	21.10	43
PI 451711	15.65	0	0.02	0.01	0.02	10.46	2.34	0.02	28.52	46
PI 477913	5.66	0	0.35	0.01	0.08	7.65	1.85	0.42	16.02	62
LSD (0.05)	NS[a]	NS	0.12	NS	0.06	NS	0.75	0.24	NS	

[a]NS, not significant.

TABLE 5

Selected Phenotypic Correlations of Tocols from *Amaranthus* spp. Tested in Three Experiments[a]

	Phenotypic correlations			
	Experiment 1 *A. cruentus*	Experiment 2 *A. hypochondriacus*	Experiment 3 *A. cruentus*	
Tocols[b]	(grain-type)	(grain-type)	Vegetable-type	Grain-type
α-T, α-T3	0.57	–0.22	–0.43	–0.44
α-T, β-T3	0.80[c]	–0.79[c]	0.77[d]	0.67
α-T, γ-T3	0.57	0.18	0.56	0.31
α-T, δ-T3	0.72[d]	NA[e]	NA	–0.93[c]
β-T3, α-T3	0.29	–0.39	–0.01	–0.14
β-T3, γ-T3	0.71[d]	0.29	0.72[d]	0.67
β-T3, δ-T3	0.56	NA	–0.62	–0.58
γ-T3, δ-T3	0.15	NA	–0.25	–0.19

[a]Means from pooled grain- and vegetable-types were used (15).

[b]T, tocopherol; T3, tocotrienol.

[c]Significance at $P \leq 0.01$.

[d]Significant at $P \leq 0.05$.

[e]Correlation unavailable due to lack of a tocol.

15.65 mg/kg seed with the other tocopherols contributing a maximum of 0.50 mg/kg seed to total tocopherols. Bertoni and Cattaneo (9) previously had found total T from 7.56 to 7.89 mg/kg seed in two CRU accessions. Most common cereal grains, except barley, contain significant quantities of either β- or γ-tocotrienols (5); thus, excluding the lack of β-T, amaranths more closely resembled tocols composition of barley than that of wheat, oats or rye. The tocols levels determined here may have been influenced by storage. Storage effects on tocols are known from maize, where stored maize lost up to 10% of its tocols (17), and from barley, where stored barley exhibited interconversion of tocols due to methylation and saturation (18–21).

Because amaranths were variously utilized as grains, vegetables or forages during the last seven centuries, artificial selection and storage may have influenced their tocol chemistry, too. For example, δ-T3, putative precursor of the other seven tocols (19–21), was found in most Mesoamerican CRU grain-types of Experiment 3, but was often absent in CRU African vegetable-types. Further research would be needed to determine whether differences in δ-T3 and other tocol levels were due to genetic drift, mutation, selection, storage or other phenomena.

The pathways for biosynthesis of tocols are currently not well understood. In plants, synthesis of δ-tocotrienol as the initial precursor has been postulated in one biosynthetic pathway (19–22). This compound is synthesized *in vivo* by the condensation of homogentisic acid and geranylgeranyl pyrophosphate (19). The γ-, β- and α-tocotrienols are formed from the δ isomer by successive methylation on the 5 and 7 positions of the chroman ring. Corresponding tocopherols are formed by the reduction of double bonds in the phytyl tails (19–22). α-Tocopherol is the end product of tocol biosynthesis. This pathway has been worked out in *Hevea brasilinensis* (19,20).

In order to better understand the relationships among the various tocol forms, we examined pooled phenotypic correlations between tocols in amaranth CRU vegetable-types and in grain-types of both species (Table 5). Curiously, HYP and CRU grain-types exhibited opposite signs in significant phenotypic correlations for α-T and β-T3. Using multiple linear regressions of proposed tocol precursors, variation in α-T was consistently explained by variation in tocols other than α-T (Lehmann, J., unpublished data, 1992). In all three experiments, backwards and stepwise regressions showed that β- and γ-T3 contributed to high coefficient of multiple determination values in equations predicting α-T variation. Statistical evaluations do not reveal intra-pathway substrates for transmethylation or hydrogenation; rather, the pathway(s) and their substrates might be elucidated by radioisotope studies (23).

Implications. Whereas nutritionally valuable protein complementation occurs between grains such as maize and amaranth (24), beneficial tocol complementation or supplementation may also happen. Notably, γ-T3, a plentiful vitamin E analogue in the HYP grains tested and in some corn inbreds (17), has been shown to inhibit cholesterol synthesis in humans (25). Compared to reported values for other grains, amaranth has a relatively high ratio of T3 types to tocols (T plus T3). This ratio is a rough measure of a grain's cholesterol suppression when 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (EC 1.1.1.34) is involved (1); here amaranths ranged from 39 to 74% T3 as a percentage of total tocols, and thus they resemble the more cholesterol-suppressive grains like barley (>70%) and oats (>80%) (1). The variation could be due to natural variation in germplasm and to interconversion of tocol isomers during storage. Amaranths of varied T3 compositions were recently tested in chickens, and these accessions lowered low density lipoprotein-cholesterol and regulated an hepatic enzyme (Qureshi, A.A., and Lehmann, J.W.,

unpublished data, 1991). Similarly, Danz and Lupton (26) found that CRU lowered serum cholesterol in rats when it was fed as a fiber supplement.

Like oat flours with a high oil content for grains, flours milled from amaranth seed or popped amaranth are often oxidatively unstable. The concentration of tocols, particularly the T3 isomers, was found to be higher in popped amaranth than in seed. This might indicate that tocols are most probably protein bound or phosphorylated on the phenolic group of the molecule (Qureshi, A.A., unpublished data, 1992). An amaranth accession such as CRU var. PI 477913, with its higher levels of γ- and δ-tocols, could be used to stabilize amaranth products; however, the relative contribution of enzymic oxidation would need to be assessed.

Taken together, the data suggest that the amaranths tested contain genetic variability for some tocols (excluding β-T) and that certain accessions contain γ- and δ-T3. Because relatively higher T3 levels were present in fresh vs. stored grains, storage effects on tocol composition are suspected. High T3 levels in stored grains with high fat content are consistent with Green's hypothesis (27), wherein non-α-tocopherols act as more effective *"in vitro"* antioxidants than α-tocopherol and thus protect the stored fat. Amaranths with differing tocol levels merit further study for nutritional or medicinal applications.

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A ^{31}P Nuclear Magnetic Resonance Investigation of Acyl Group Transfer from Phosphatidylcholine to Yield Lysophosphatidylcholine in Human Plasma

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^{31}P nuclear magnetic resonance (NMR) spectroscopy was used to measure the rate of acyl transfer from phosphatidylcholine (lecithin, PC) in whole plasma and in high density lipoprotein (HDL). Spectral deconvolution was used to resolve overlapping resonances in the ^{31}P NMR spectra of the phospholipids. Mean values of the acyl group transfer rates from PC in plasma and HDL were 36 μmol $L^{-1}h^{-1}$ and 19 μmol $L^{-1}h^{-1}$, respectively. The reciprocal nature of the decrease in the spectral peak intensities of PC, compared to the increase in the intensities of the lysolecithin (lysoPC) peaks, suggested a substrate/product relationship consistent with the action of lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for the esterification of free cholesterol in plasma. LCAT involvement was confirmed by measuring the cholesterol esterification rate based on the ^{13}C NMR spectra obtained on lipid extracts from plasma that had been incubated at 37°C. Within experimental error, the rate of lysoPC formation in plasma was shown to be equal to that of cholesteryl ester formation.

Lipids 29, 183–188 (1994).

The transfer of a fatty acid residue from the *sn*-2 position of phosphatidylcholine (lecithin, PC) onto carbon-3 of cholesterol in human plasma is catalyzed by lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) (1–3). The reaction products are cholesteryl ester and lysophosphatidylcholine (lysolecithin, lysoPC). The reaction typically utilizes high density lipoprotein (HDL) (4,5), which contains apolipoprotein A-I, an activator of LCAT (6–8), although there is also evidence that the enzyme can act directly on low density lipoprotein (LDL) (9). The cholesteryl ester newly synthesized in HDL is then transferred to, and exchanged between, very low density lipoprotein and LDL, replacing triglyceride (9,10). This maintains a concentration gradient for the flux of additional unesterified cholesterol from cellular membranes and from other lipoproteins to HDL. The exchange process is mediated by a glycoprotein, namely cholesteryl ester transfer protein (CETP) (10–13).

The most widely used clinical biochemical methods for measuring cholesterol esterification rates, or cholesteryl ester transfer activity, use radioactive tracers. In these assays, a radiolabelled cholesterol/albumin emulsion is added to the plasma or lipoprotein fraction and is pre-incubated at 4°C to allow equilibration of the labelled cholesterol with endogenous free cholesterol (14) before incubation to achieve acyltransfer at 37°C. After extraction and separation of the cholesterol and the cholesteryl ester fractions, the radioactivity associated with cholesteryl esters is measured by liquid scintillation counting (14).

We have developed a ^{31}P nuclear magnetic resonance (NMR) method to measure the rate of conversion of PC to lysoPC in human plasma and HDL. The method obviates the need to add exogenous substrates and to extract and separate the products. The reciprocal relationship we observed between the rate of decrease in PC and the rate of increase in lysoPC during plasma incubation suggested a substrate/product relationship consistent with the action of LCAT, the enzyme responsible for esterification of free cholesterol in plasma. The rate of lysoPC formation was also shown to be directly correlated with the formation of cholesteryl ester in plasma based on natural abundance ^{13}C NMR analyses on plasma extracts. The ^{31}P NMR method provides a simple, one-step means to measure the rate of acyl group transfer from PC to cholesterol in plasma. The LCAT reaction rates we measured were very similar to those that have been obtained by traditional methods.

MATERIALS AND METHODS

Materials. PC and lysoPC, both from egg yolk, as well as cholesterol, sphingomyelin, cholesteryl palmitate and dihydrocholesterol, were obtained from Sigma Chemical Co. (St. Louis, MO). D_2O (99.75%) was from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, NSW, Australia). $CDCl_3$ was from Merck, Sharpe & Dohme Isotopes (Montreal, Quebec, Canada). All other reagents were analytical grade. Tris-HCl (0.1 M) containing 140 mM NaCl, pH 7.4, and 20% D_2O (vol/vol) served as pH buffer.

Plasma samples. Pooled plasma was obtained from outpatients at the Royal Prince Alfred Hospital (NSW, Australia), who were there for conditions other than cardiovascular disease. Subjects were excluded if they had suffered any acute illness during the previous 3 mon, or if they had any condition or treatment which was likely to affect lipid metabolism. Venisection was performed following a 12-h, overnight fast. Blood samples were anticoagulated with ethylenediaminetetraacetic acid (0.1% wt/vol final concentration), and plasma was separated immediately by centrifugation at 3000 × *g* for 10 min at 4°C.

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Abbreviations: CETP, cholesteryl ester transfer protein; DTNB, 5,5-dithio*bis*(-2-nitrobenzoic acid); GPC, glycerophosphorylcholine; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; lysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; SPH, sphingomyelin.

Preparation of HDL. HDL was isolated following precipitation of lipoproteins containing apolipoprotein B according to the method of Warnick *et al.* (15).

Sample preparation. Plasma and HDL were concentrated over 3–9 h at 4°C by use of a Minicon concentration (W.R. Grace, Danvers, MA). After concentration (about threefold), 2.5 mL of plasma or HDL was transferred to a 10-mm NMR tube, and the volume was brought up to 3 mL with D_2O Tris buffer, pH 7.4 (see Materials and Methods section). The samples were then ready for incubation and NMR measurements. Standard samples were prepared by adding a known concentration of lysoPC in Tris buffer to plasma.

Extraction of lipids. Total lipids of plasma (10 mL) were extracted according to the method of Bligh and Dyer (16), and dried under a stream of nitrogen. Dried lipids were then dissolved in 3 mL of $CDCl_3$ for NMR measurements.

NMR spectroscopy. All ^{31}P NMR spectra were acquired under identical conditions on a Bruker AMX-400 NMR spectrometer (Karlsruhe, Germany) operating at 161.98 MHz in the Fourier transform mode; probe temperature was kept at 37 ± 1°C. Broadband proton decoupling was applied to eliminate ^{1}H-^{31}P peak multiplicities. All measurements were done on 3-mL samples in 10-mm NMR tubes with D_2O as internal field/frequency lock. Samples were spun at 12 Hz, and a 15 µs (90°) pulse, a 1 s pulse delay, a 0.67 s acquisition time, 16k data points and 256 transients were used. Free induction decays (FID) were processed with 6 Hz line broadening to improve apparent signal-to-noise ratio. ^{31}P and ^{13}C NMR measurements on plasma extracts were done on $CDCl_3$ solutions with $CDCl_3$ also serving as the internal field/frequency lock; the probe temperature was 25 ± 1°C. ^{13}C NMR spectra were acquired at 100.61 MHz, using the method by Homer and Roberts (17). Spectra were derived by Fourier transformation of 2124 summed transients, and FID were processed with 1 Hz line broadening.

Quantification of lipids. Automatic Lorentzian deconvolution was used to resolve overlapping phosphorus resonances of PC and lysoPC. To measure reaction rates, areas of deconvoluted ^{31}P NMR spectra that were obtained on plasma extracts at different time points were quantified, and the areas were compared with those obtained for known concentration of authentic compounds. However, for measurements on plasma and HDL, peak heights rather than peak areas were used, for quantification, because small changes in the widths of the phospholipid phosphorus resonances were observed in the course of the LCAT reaction and good results were obtained based on peak height measurements.

Free cholesterol and cholesteryl esters were quantified based on peak areas of the ^{13}C NMR resonances for carbon-3 of these lipids relative to carbon-3 of dihydrocholesterol used as the internal standard. The differences between the peak areas of dihydrocholesterol used as internal standard and those of cholesterol and cholesteryl ester, due to different relaxation rates of the respective nuclei, were corrected by a coefficient that was determined by addition of known amounts of standard samples and by measuring the areas of the resulting ^{13}C NMR signals. Peak areas were determined using automatic Lorentzian deconvolution rather than direct integration, because small overlapping resonances were seen in the spectra.

RESULTS

^{31}P NMR on plasma. A ^{31}P NMR spectrum obtained on fresh human plasma is shown in Figure 1A. The resonances seen include those due to PC at −0.31 ppm, lysoPC at 0.15 ppm, and inorganic phosphate at 2.9 ppm. These assignments were confirmed by adding authentic PC, as liposomes, as well as lysoPC to the plasma as internal standards. Chemical shift assignments are referenced relative to methylenediphosphonic acid, kept in a coaxial capillary with a signal at 16.89 ppm relative to 85% orthophosphoric acid at 0.000 ppm (18).

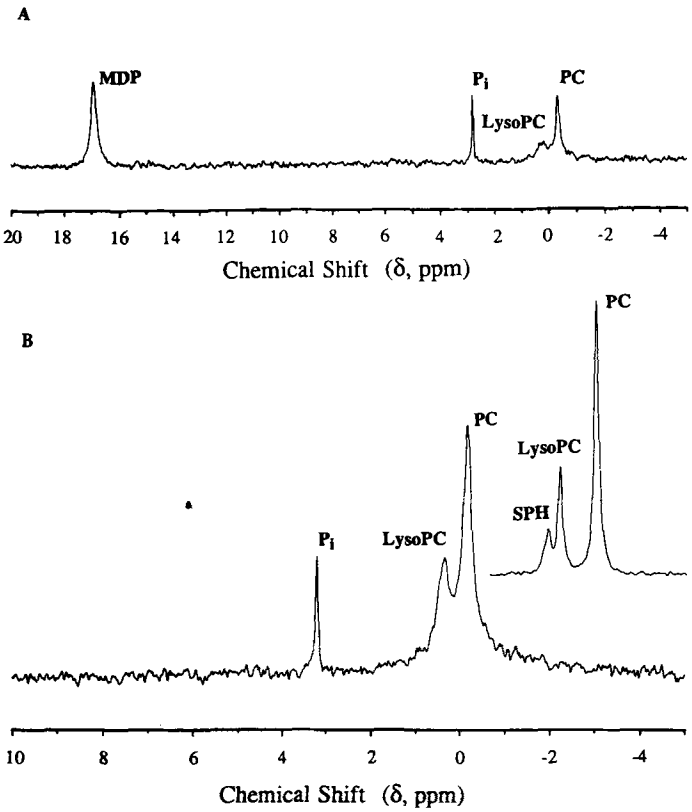

FIG. 1. A, ^{31}P nuclear magnetic resonance (NMR) spectrum of human plasma (2 mL nonconcentrated plasma with 1 mL 0.1 M Tris buffer, pH 7.4 at 25°C). Resonance assignments were confirmed by adding lecithin [phosphatidylcholine (PC)] liposomes and lysolecithin [lysophosphatidylcholine (lysoPC)] to the plasma sample. The chemical shifts were referenced relative to methylenediphosphonic acid (MDP) at 16.89 ppm. P_i denotes inorganic phosphate. B, ^{31}P NMR spectrum of threefold concentrated plasma and a total lipid extract of plasma (plasma was incubated for 5 h prior to extraction) in $CDCl_3$ (A, chemical shift scale offset, ≈3 ppm). Resonance assignments for the phospholipids in the plasma extract were confirmed by adding authentic PC, lysoPC and sphingomyelin (SPH) to the solution of the plasma extract.

The phosphorus resonances obtained on plasma (e.g., Fig. 1A) can be of low intensity and can be difficult to fully resolve. This difficulty can be overcome by either using a greater number of transients at the expense of accumulation time, or by increasing the sample concentration. A ^{31}P NMR spectrum of threefold concentrated plasma is shown in Figure 1B. As can be seen, the amplitudes of the signals obtained under otherwise identical spectral conditions are significantly increased when compared with those obtained on native plasma. In Figure 1B, the top insert shows a ^{31}P NMR spectrum of a total lipid extract from plasma in $CDCl_3$ in which the sphingomyelin (SPH) resonance is distinguishable from the lysoPC peak, and the PC resonance is fully resolved.

Plasma and HDL time course. Figure 2A shows the ^{31}P NMR signals obtained on 1.85-fold concentrated plasma samples over a time course of about 16 h. The incubation temperature was 37°C, and the reaction was monitored by recording sequential spectra at the time points indicated. A plot of the amplitudes of the PC and lysoPC peaks obtained on the deconvoluted spectra, as shown in Figure 2A, vs. time, is given in Figure 2B. For this and similar time courses, regression analyses indicated that the slopes of the curves for PC decline and lysoPC increase indicated very similar reaction rates. Figure 3 shows a plot of lysoPC concentrations in 2.77-fold concentrated HDL, incubated at 37°C, and of those in concentrated plasma. As can be seen, the increase of lysoPC concentration in plasma (37 ± 3.8 μmol $L^{-1}h^{-1}$) was faster than that in HDL (15 ± 3.2 μmol $L^{-1}h^{-1}$).

Acyl transfer activity, which is reflected in the rate of lysoPC formation, was calculated from the initial slope of the lysoPC concentration increase in concentrated plasma and in HDL. In Table 1 the results of six separate experiments used for the determination of acyl transfer activity from PC in plasma are presented. Also, in three of these experiments, the acyl transfer rate in HDL isolated from the same plasma was measured.

The effect of plasma concentration. To assess the effect of plasma concentration on the measured reaction rate, parallel time-course experiments were carried out with concentrated and native plasma samples. In order to improve the signal-to-noise ratio of the spectra, the number of transients was increased from the 256 used for concentrated plasma samples to 1280 (5-fold) for nonconcentrated plasma samples. The rates of acyl transfer measured were not significantly different after correcting (scaling) for the different concentrations used (Table 1). Thus, the use of concentrated plasma permits more precise estimates of reaction rates within a much shorter period of time.

^{13}C NMR study. Experiments were carried out to directly compare the formation of lysoPC and of cholesteryl ester, i.e., acyl transfer due to LCAT activity. The plasma samples were incubated at 37°C, extracted, and

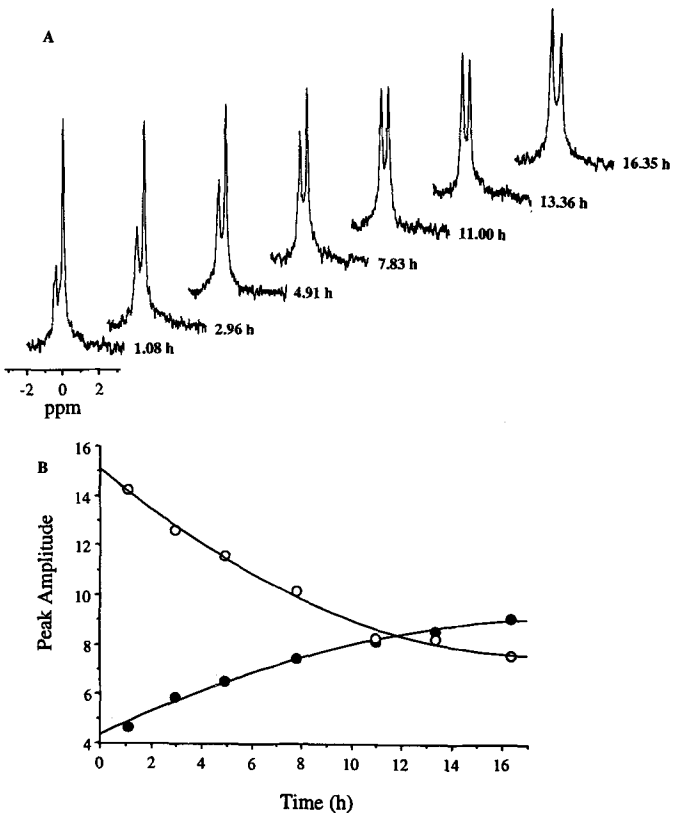

FIG. 2. A, sequential ^{31}P NMR spectra of 1.85-fold concentrated plasma incubated at 37°C for the times indicated. B, plot of PC (open circles) and lysoPC (closed circles) peak amplitudes (arbitrary units) derived from deconvolutions of the spectra shown in A. The decline in PC peak intensities mirrors the increase in lysoPC peak intensities. The curves were drawn using an empirical quadratic polynomial $y = a + bt + ct^2$, where t is the time and the fitted parameters for lysoPC and PC had the values a = 4.326, b = 0.497, c = -1.282×10^{-2}, and a = 15.107, b = −0.846, c = 2.382×10^{-2}, respectively. Abbreviations as in Figure 1.

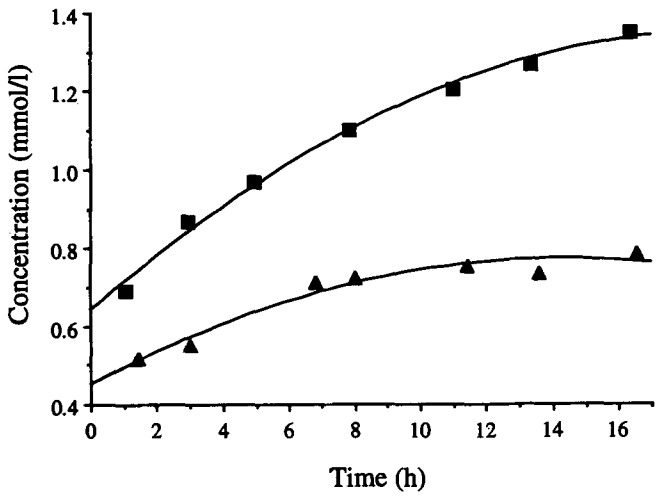

FIG. 3. Comparison of lysoPC concentrations derived from deconvoluted ^{31}P NMR spectra of 2.77-fold concentrated high density lipoprotein (HDL) that was incubated at 37°C (triangles), and of plasma as derived from Figure 2A (squares). The curves were drawn using an empirical quadratic polynomial $y = a + bt + ct^2$. Fitted parameters for HDL and plasma lysoPC concentration increase had the values a = 0.450, b = 4.485×10^{-2}, c = -1.563×10^{-3}, and a = 0.640, b = 7.350×10^{-2}, c = -1.895×10^{-3}, respectively. Abbreviations as in Figure 1.

TABLE 1

Rates of Acyl Transfer (μmol L^{-1} h^{-1}) from Phosphatidylcholine in Plasma and HDL[a]

Experiment	Concentrated plasma	Nonconcentrated plasma	HDL[b]
1	39 ± 4.2	37 ± 7.4	
2	42 ± 4.3	40 ± 6.0	
3	36 ± 7.7		
4	37 ± 3.8		15 ± 3.2
5	48 ± 19		25 ± 4.5
6	32 ± 2.8		20 ± 1.8
Weighted means[c]	36	39	19

[a]The rates of acyl transfer were calculated from the slopes of the plot of lysophosphatidylcholine (lysoPC) concentration vs. incubation time in concentrated and nonconcentrated plasma, and in high density lipoprotein (HDL), in six separate experiments. LysoPC concentrations were derived from sequential ^{31}P nuclear magnetic resonance spectra over time, as shown in Figures2A. The concentration of lysoPC in concentrated plasma and HDL was calculated as μmol (L plasma)$^{-1}$ of lysoPC in the initial sample (before concentration). Values are means ± SD.

[b]HDL was isolated by precipitation of apo B-containing lipoproteins, and was then concentrated (see Materials and Methods section).

[c]We used the "statistical weight," 1/variance.

TABLE 2

Cholesterol Esterification Rate (μmol L^{-1} h^{-1}) in Human Plasma

Experiment	Cholesterol esterification rate[a]
1	55 ± 9
2	53 ± 4
3	32 ± 12
Weighted mean[b]	51

[a]The cholesterol esterification rate was calculated from the initial slope of the plot of cholesteryl ester concentration in plasma vs. incubation time. Cholesteryl ester concentration was derived from sequential ^{13}C nuclear magnetic resonance spectra of total lipid extracts of plasma over time (see Materials and Methods section). Values of rate constants are means ± SD and are expressed in μmol (L plasma)$^{-1}$ h^{-1}.

[b]We used the "statistical weight," 1/variance.

the products measured by ^{31}P and ^{13}C NMR. The cholesterol esterification rate was calculated from the initial slope of either the cholesteryl ester concentration increase or the free cholesterol concentration decline. Separate time courses were obtained by ^{31}P and ^{13}C NMR, and the rates of acyl transfer from PC to yield lysoPC and cholesterol ester were measured. In a typical example, for plasma from a single donor, the rates based on lysoPC and cholesteryl ester formation were 41 ± 9 μmol $L^{-1}h^{-1}$ and 32 ± 12 μmol $L^{-1}h^{-1}$, respectively. The two rates were statistically not different. This was done when the weighted mean rates in plasma of lysoPC formation (Table 1, 39 μmol $L^{-1}h^{-1}$) and of cholesteryl ester formation (Table 2; 51 μmol $L^{-1}h^{-1}$) were compared; the rates again were quite similar. As the samples were from different donors, some of the variance may actually be due to biological variations between the donors. Although the rate estimates have a relatively large standard deviation, the results obtained by ^{13}C and ^{31}P NMR corroborate each other and are consistent with acyl transfer from PC to free cholesterol catalyzed by LCAT.

DISCUSSION

Incubation of concentrated plasma at 37°C resulted in the simultaneous increase in lysoPC levels and the decrease in PC levels with similar velocities (Fig. 2), which is consistent with lysoPC formation directly from PC in the course of cholesterol esterification (4) catalyzed by LCAT activity. The rates of acyl transfer we observed in plasma *in vitro* (Table 1) were quite similar to those for cholesterol esterification by LCAT, that had been reported by others (19,20). LysoPC formation from HDL–PC (Fig. 3) accounted for an increasingly smaller proportion relative to lysoPC formation in whole plasma as the incubation progressed. This decrease in HDL was most likely due to the depletion of substrate (PC) and the accumulation of reaction products that inhibit LCAT. In whole plasma, additional substrates (PC and cholesterol) can be exchanged from other lipoproteins while the product, cholesteryl ester, is removed from HDL by CETP and possibly other carrier proteins. In addition, a smaller proportion of the increase in lysoPC concentration in plasma could be due to LCAT acting on LDL (9), which also may account, in part, for the higher apparent LCAT activity in whole plasma seen later in the incubation when compared with HDL (Fig. 3). Furthermore, LDL-activated lysoPC acylation by LCAT has been reported to occur. This transfer of an acyl group from PC in LDL to lysoPC (21,22) however, would not cause a net change in the total mass of PC or lysoPC and would therefore have no effect on the net LCAT activity measured by the present method. In addition to this transesterification reaction, the acyl group of PC can be transferred to water, which amounts to a phospholipase-like activity of LCAT (23,24). However, this reaction occurs only when cholesterol levels are very low, such as in cholesterol-deficient liposomes, and this activity is distinct from that of phospholipase A_2 (25). Brasure *et al.* (26) have previously used ^{31}P NMR to monitor the hydrolysis of phospholipids in human HDL_3 upon addition of exogenous phospholipase A_2. They observed the changes in the spectra of HDL_3 produced by digestion with snake venom phospholipase A_2 *(Crotalus adamanteus)* when incubated at 28°C and noted that PC was completely converted to lysoPC in the course of the reaction. Although Brasure *et al.* used ^{31}P NMR to monitor lipid-enzymatic changes by an approach similar to ours, these authors utilized an exogenous enzyme from a source other than human plasma. LDL and apolipoprotein B, on the other hand, have been reported to exhibit phospholipase A_2-type activity toward phospholipids containing an oxidized or a short fatty acyl chain at position-2 (27,28). This phospholipase activity, however, has been shown to produce only glycerophospho-

rylcholine (GPC) and not lysoPC, up to the complete hydrolysis of the substrate (28). In our studies, we were unable to detect any GPC resonance by ^{31}P NMR on plasma extracts, even after extended (several hours) incubation times; also GPC is usually not considered to be a major constituent of human plasma (29,30). This would not exclude, however, that such activity by LDL may be exhibited toward an exogenous substrate in an isolated or in an *in vitro* system.

For quantification of lysoPC and PC in plasma by ^{31}P NMR, peak amplitudes, rather than areas, were used because the apparent peak widths of PC and lysoPC changed with plasma incubation time. NMR line width is known to be affected by a number of parameters, including translational and rotational motions (T_2) of the nuclei involved and by inhomogeneities in the magnetic microenvironments (31). For example, for phospholipid vesicles, changes in vesicle size directly affect rotational motions and line widths (32,33), and similar differences in ^{31}P NMR linewidths have been reported for phospholipids in HDL and LDL (34). Phospholipids in plasma are continuously being exchanged between different lipoproteins of different size and lipid/protein compositions. On the other hand, the chemical shift of each class of phospholipids depends upon the composition of the particles (34,35). Thus, small changes in chemical shifts of close ^{31}P NMR resonances would also result in a change in apparent linewidths. If subsequent deconvolution involves fitting a line shape with less than the full complement of peaks, then the fit to the broadened lines will attempt to accommodate this increase and lead to larger "wings" on the fitted function, which would manifest itself as an increase in the apparent peak area. Any of these effects would make it difficult to obtain quantitative estimates from peak areas. A practical solution was to use peak heights as measure for the quantification of phospholipid concentrations in plasma. Using this approach, we demonstrated in plasma that the velocities of the increases in lysoPC levels and decreases in PC levels were the same within experimental error.

The phosphorus resonances in the ^{31}P spectra of concentrated plasma (Fig. 1B, lower) can be primarily attributed to PC, lysoPC and SPH, of which the resonances of lysoPC and SPH are more difficult to resolve. Earlier ^{31}P NMR studies on phospholipids and estimates of phospholipid concentrations in plasma have been published (29,30,36,37), although our study is the first whose aim was to develop, evaluate and apply an NMR-based LCAT assay.

Standard procedures for measuring the rate of cholesterol esterification and transfer from HDL typically involve radiolabelling. For this purpose, plasma or lipoprotein fractions are first preincubated with a radioactive cholesterol/albumin mixture to allow equilibration between labelled exogenous cholesterol and endogenous native cholesterol. In most of these assays, LCAT is first inhibited with 5,5-dithio*bis*-2-nitrobenzoic acid (DTNB), and the inhibition is then reversed by incubation with mercaptoethanol (14). The cholesterol esterification and transfer rates are then estimated by measuring, after chromatographic separation, the radioactivity that is associated with cholesteryl ester in plasma or in the lipoprotein fraction. Although these assay methods are used extensively, they suffer from several disadvantages: (i) cholesterol esterification is not completely inhibited by DTNB during the preincubation period, and mercaptoethanol may alter the esterification rate (14); (ii) complete equilibration of radioactive cholesterol with endogenous cholesterol during preincubation is uncertain, and it is difficult to determine whether the labelled cholesterol has been distributed uniformly among the lipoproteins; and (iii) other assays of LCAT activity or cholesteryl ester transfer activity with artificial substrates, such as PC/cholesterol liposomes, may not necessarily be representative of the activities *in vivo*.

In conclusion, the novel ^{31}P NMR procedure we developed for measuring the rate of acyl transfer from PC to free cholesterol in plasma, or HDL, mediated by LCAT, has a number of advantages for assaying LCAT activity. The introduction of NMR spectroscopy into clinical biochemistry should entail the development of ancillary procedures to replace older methods, or the development of new procedures for assaying enzyme activities and metabolites that have proven intractable by other means.

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Garlic Reduces Plasma Lipids by Inhibiting Hepatic Cholesterol and Triacylglycerol Synthesis

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Prompted by the reported hypolipidemic activity of garlic, the present study was undertaken to elucidate the mechanism(s) underlying the cholesterol-lowering effects of garlic. Rat hepatocytes in primary culture were used to determine the short-term effects of garlic preparations on [1-^{14}C]acetate and [2-^{3}H]glycerol incorporation into cholesterol, fatty acids and glycerol lipids. When compared with the control group, cells treated with a high concentration of garlic extracts [i.e., petroleum ether- (PEF), methanol- (MEF) and water-extractable (WEF) fractions from fresh garlic] showed decreased rates of [1-^{14}C]acetate incorporation into cholesterol (by 37–64%) and into fatty acids (by 28–64%). Kyolic containing *S*-allyl cysteine and organosulfur compounds inhibited cholesterogenesis in a concentration dependent manner with a maximum inhibition of 87% at 0.4 mM. At this concentration, Kyolic decreased [1-^{14}C]acetate incorporation into fatty acids by 67%. *S*-allyl cysteine at 2.0 and 4.0 mM inhibited cholesterogenesis by 20–25%. PEF, MEF and WEF depressed the rates of [2-^{3}H]glycerol incorporation into triacylglycerol, diacylglycerol and phospholipids in the presence of acetate, but not in the presence of oleate. The results suggest that the hypocholesterolemic effect of garlic stems, in part, from decreased hepatic cholesterogenesis, whereas the triacylglycerol-lowering effect appears to be due to inhibition of fatty acid synthesis. Primary hepatocyte cultures as used in the present study have been proven useful as tools for screening the anticholesterogenic properties of garlic principles.

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Aside from its general use as a condiment, garlic (*Allium sativum*) is known for its pharmacological and nutritional properties (1). Among the potential therapeutic applications of garlic, its antilipidemic effects have been demonstrated in studies on humans (2–6), as well as on animals, including rats (7–13), rabbits (14,15), chicken (16–17) and swine (18). Garlic is known to contain a variety of sulfur compounds, in addition to amino acids, vitamins and minerals (19). Although some of these sulfur compounds, such as *S*-methylcysteine sulfoxide and *S*-allyl cysteine sulfoxide, are known to reduce cholesterol levels in the liver and plasma (10), the principle(s) foremost responsible for the hypolipidemic action of garlic is presently not known. Likewise, the mechanism(s) underlying the hypocholesterolemic effects of garlic constituents(s) have not been fully elucidated (20).

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Abbreviations: DME, Dulbecco's modified Eagle medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); FBS, fetal bovine serum; MEF, methanol-extractable fraction of garlic; PEF, petroleum ether-extractable fraction of garlic; PL, phospholipid; TG, triacylglycerol; WEF, water-extractable fraction of garlic.

The liver is a major site of cholesterol and lipoprotein synthesis and of cholesterol clearance *via* low density lipoprotein-receptor mediated processes (21,22). Previous studies by other investigators have shown that garlic supplemented diets can depress the activities of various lipogenic enzymes including glucose-6-phosphate dehydrogenase and malic enzyme (9,10,17), as well as of the cholesterogenic enzyme, 3-hydroxy-3-methyl-glutaryl-CoA reductase (16–18). It therefore seems reasonable to postulate that the hypolipidemic effect of garlic results from impaired lipogenesis and cholesterogenesis in the liver.

The goal of the present study was to determine whether garlic suppresses cholesterol and glycerol lipid biosynthesis in hepatocytes in primary culture. The results we obtained demonstrated that extracts prepared from fresh and aged garlic exert a marked inhibition on cholesterol and fatty acid synthesis.

MATERIALS AND METHODS

Chemicals. Radioactive substrates were purchased from Amersham Corp. (Arlington Heights, IL). Collagenase D was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Culture media, fetal bovine serum (FBS) and antibiotics were the products of GIBCO (Gaithersburg, MD). Aged garlic powder, Kyolic (liquid form of aged garlic), and *S*-allyl-cysteine were provided by Wakunaga of America Co., Ltd. (Mission Viejo, CA). Substrates and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Diet ingredients were supplied by ICN Biochemicals (Costa Mesa, CA).

Animals and diets. Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and fed a nonpurified diet (Purina Rat Chow; Ralston Purina, St. Louis, MO). The animals were housed individually in stainless steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (0600–1800). In experiments on the effects of garlic on plasma lipids, two groups of five animals each (140–150 g body weight) were fed high-fat diets (20 g corn oil/100 g diet), and one group was fed the diet supplemented with 2% aged garlic powder. Thus, the compositions of the two diets were the same, except for the presence of garlic in the experimental group (Table 1). The diets were isocaloric and provided 98.3 kJ/100 g diet. The diets were stored under nitrogen at –20°C and provided fresh to the animals daily. Unconsumed portions of the diets were discarded each day.

Hepatocyte culture. Liver cells were isolated from rats (200–250 g body weight) according to the method of Berry and Friend (23), as modified by Seglen (24). Rats were anesthetized with nembutal (5 mg/100 g body weight), and the hepatic portal vein was cannulated for perfusion with buffer [NaCl, 142 mM; KCl, 6.7 mM;

TABLE 1

Composition of Control and Experimental Diets[a]

Ingredient	Control (g/kg diet)	Experiment (g/kg diet)
Casein	172	172
Sucrose	203	203
Corn starch	295	295
Corn oil	200	200
AIN-76 mineral mix	35	35
AIN-76A vitamin mix	10	10
Fiber, cellulose	50	48
Choline, dihydrogen citrate	2	2
L-Methionine	3	3
Agar	30	30
Aged garlic powder[b]	0	2

[a]All diet ingredients were purchased from ICN Biochemicals (Costa Mesa, CA). Both diets contained the same energy density, i.e., 98.3 KJ/100 g.

[b]Aged garlic powder was provided by Wakunaga of America Co., Ltd. (Mission Viejo, CA).

N-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 10 mM; NaOH, 5.5 mM; pH 7.4] for 15 min. Immediately after perfusion *in situ*, the liver was carefully excised and perfusion with collagenase buffer (NaCl, 67 mM; KCl, 6.7 mM; HEPES, 100 mM; $CaCl_2 \cdot H_2O$, 5.4 mM; NaOH, 66 mM; pH 7.6; 50 mg collagenase D/100 mL) was continued for 15 min. The enzyme-treated liver was then subjected sequentially to mincing, incubation, filtration and centrifugation for cell isolation and purification. From each liver, 100–250 $\times 10^6$ cells were obtained with a viability of 95–98% as judged by trypan blue exclusion. The cells were resuspended in Dulbecco's modified Eagle medium (DME) supplemented with 5.6 mM glucose, 10% FBS and antibiotics (100 units penicillin/mL, and 100 μg streptomycin/mL) to obtain 0.8–1.0 $\times 10^6$ cells/mL of suspension. Two-mL aliquots of the suspension were plated per well in a six-well culture plate (Becton Dickinson Labware, Lincoln, NJ) and incubated at 37°C under an atmosphere of 95% air and 5% CO_2. After 6 h of incubation, nonadhering cells were removed and discarded. Hepatocytes that adhered to the culture plate were refed with DME and incubated for24 h.

Metabolic studies. At the end of the 24-h incubation, cells were washed three times with 2 mL of FBS-free DME, followed by incubation with 2 mL of the same medium containing [1-^{14}C]acetate (specific activity, 37 MBq/mmol) and 0.5 mM nonlabeled sodium acetate, or [2-^{3}H]glycerol (specific activity, 18.5 MBq/mmol) and 0.1 mM nonlabeled glycerol in the presence or absence of the agents to be tested, i.e., garlic extracts, Kyolic and *S*-allyl cysteine. After 4 h of incubation, the medium was collected and cells were harvested with 1.3 mL of ice-cold water by scraping with a rubber policeman. [1-^{14}C]Acetate and [2-^{3}H]glycerol were used to estimate cholesterogenic and lipogenic rates (25).

Lipid analysis. The harvested cells were mixed with 20 mL of chloroform/methanol (2:1, vol/vol) to extract the lipids according to the method of Folch *et al.* (26). For measurement of [1-^{14}C]acetate incorporation into cholesterol and fatty acids, the lipid extract was saponified in 6 mL of 3.75% methanolic KOH at 90°C for 4 h (27). Nonsaponifiable lipids were extracted with petroleum ether (b.p. 35–60°C), and cholesterol was precipitated with digitonin (27). After removal of the nonsaponifiable fraction, the extracts were acidified with concentrated HCl, and the fatty acids were extracted with petroleum ether. For quantification of [2-^{3}H]glycerol incorporation into glycerol lipids, lipids were separated into triacylglycerols (TG), diacylglycerols and phospholipids (PL) by thin-layer chromatography on Silica Gel G coated plates using hexane/diethyl ether/acetic acid (80:20:1, by vol) as developing solvent (28). The radioactives of ^{14}C- and ^{3}H-labeled products were measured by liquid scintillation counting (Beckman Model LS 6800; Beckman Instruments, Houston, TX).

Preparation of garlic extracts. Fresh garlic purchased from a local market was used in the study. Husk-free garlic bulbs (100 g) were homogenized with petroleum ether to obtain a petroleum ether-extractable fraction (PEF) according to the method of Qureshi *et al.* (17). Semiliquid residues that remained after petroleum ether extraction were freeze-dried for 72 h and yielded 36.6 g of dry powder. Two portions of 10 g each were used for preparation of methanol- (MEF) and water-extractable (WEF) fractions, as described elsewhere (17). The final preparations of PEF, MEF and WEF were resuspended in ethanol, methanol and water, respectively. All extracts were sterilized by microfiltration through 0.22 μm cellulose acetate filters. The concentrations of PEF, MEF and WEF used for the metabolic studies were adjusted so that 1 × and 5 × extracts were equivalent to 0.25 mg and 1.25 mg of dried powder, respectively. Five microliters of the extract were added to 2 mL of the incubation medium.

Statistics. Data are presented as means ± SE, and the difference in values between control and experimental groups in the *in vivo* study was analyzed by Student's *t*-test. The comparisons of the test compounds *in vitro* were made by one-way analysis of variance. When statistical significance was indicated by the analysis of variance, Duncan's Multiple Range test was used to identify the significant difference between the groups at $P < 0.05$.

RESULTS

Prior to our *in vitro* studies, a feeding experiment was carried out to confirm the hypolipidemic effects of garlic that have been observed by other investigators in different animal species (7–18). One group of rats was fed a high-fat diet containing 2% aged garlic powder, and the other group received a similar diet without garlic for four weeks. As shown in Table 2, at the conclusion of the feeding experiment there was no difference in body weights between the two groups of rats on the two diets. The fasting plasma TG and total cholesterol concentrations in the garlic group were lowered by 30 and 15%, respectively, relative to those in the group fed the garlic-free diet. High density lipoprotein cholesterol levels

TABLE 2

Hypolipidemic Effects of Aged Garlic Powder in Rats[a]

	Control diet	2% Garlic diet
Body weight (g)	309 ± 6	317 ± 12
Plasma (mmol/L)		
Triacylglycerol	0.90 ± 0.07	0.63 ± 0.02[b]
Cholesterol	2.38 ± 0.10	2.02 ± 0.03[b]
HDL[c] cholesterol	1.78 ± 0.10	1.73 ± 0.03

[a]Values are means ± SE for five animals. Animals were fasted for 12 h before blood sampling.

[b]Indicates a significant difference from the corresponding value of the control group at $P < 0.05$.

[c]High density lipoprotein.

were the same in the two groups. These findings led us to the subsequent *in vitro* experiments that were aimed at investigating the mechanism(s) that would underlie the cholesterol-lowering effect of garlic.

The rates of hepatic *de novo* fatty acid and cholesterol synthesis were determined using [1-^{14}C]acetate as substrate. The rate of [1-^{14}C]acetate incorporation into cholesterol was lowered 36% by PEF and 44% by MEF at the 5 × concentration (Table 3). No significant inhibition by these extracts was seen at the 1 × concentration. On the other hand, WEF at 1 × lowered acetate incorporation by 52%, but an increase of the concentration to 5 × did not further reduce the rate of incorporation. WEF and MEF were equally effective in inhibiting [^{14}C]acetate incorporation into fatty acids. Inhibition at 1 × and 5 × concentrations ranged from 59 to 64%. The rate of fatty acid synthesis was inhibited by PEF at 5 ×, but not at 1 × concentration.

To further characterize the inhibitory effect of garlic constituents on lipid biosynthesis, Kyolic and *S*-allyl cysteine were tested for their effectiveness. *S*-Allyl cysteine is a major sulfur-containing amino acid derivative in garlic. Kyolic preparation used in the study contained 700 mg *S*-allyl cysteine/L (equivalent to 4.3 mM) and other, not specified, water-soluble compounds. The rate of [1-^{14}C]acetate incorporation into cholesterol was not influenced by *S*-allyl cysteine at concentrations below 1.0 mM (Table 4). At high concentrations (i.e., 2.0 and 4.0 mM), the rates were 20–25% lower than those in the controls. Fatty acid synthesis from [1-^{14}C]acetate was not altered by *S*-allyl cysteine at all concentrations tested. Kyolic at a concentration equivalent to 0.05 mM *S*-allyl cysteine was effective in reducing the rate of [1-^{14}C]acetate incorporation into cholesterol, and the inhibition was shown to be concentration dependent (Table 4). At a higher concentration equivalent to

TABLE 3

Inhibition of [1-^{14}C]Acetate Incorporation into Lipids of Hepatocytes by Garlic Extracts[a]

Treatment[b]	[1-^{14}C]Acetate incorporation into	
	Cholesterol	Fatty acid
	(pmol acetate incorporated/mg protein/4 h)	
Control	120 ± 11[c]	704 ± 55[c]
PEF		
1 ×	108 ± 11[c]	642 ± 23[c]
5 ×	77 ± 12[d]	500 ± 16[d]
MEF		
1 ×	102 ± 8[c]	270 ± 19[e]
5 ×	67 ± 10[d]	291 ± 35[e]
WEF		
1 ×	57 ± 8[d]	253 ± 25[e]
5 ×	43 ± 4[d]	270 ± 16[e]

[a]Hepatocytes were incubated in 2 mL serum-free Dulbecco's modified Eagle medium containing 0.5 mM [1-^{14}C]acetate (specific activity, 1.0 mCi/mmol or 37 MBq/mmol).

[b]PEF (petroleum ether), MEF (methanol) and WEF (water)-extractable fractions of fresh garlic were resuspended in ethanol, methanol and water, respectively; 1 × extract was equivalent to 0.25 mg and 5 × to 1.25 mg of dry garlic powder. Five μL of the extracts was added to the incubation medium.

[c–e]Values are means ± SE of three experiments. The average of three wells measured in each experiment was considered a data point. When compared with the control group, addition of 5 μL ethanol decreased the incorporation of [1-^{14}C]acetate into cholesterol by 26 pmol/mg protein/4 h and increased the incorporation into fatty acid by 162 pmol/mg protein/4 h. On the other hand, addition of 5 μL methanol reduced the incorporation into cholesterol and fatty acid by 17 pmol and 63 pmol/mg protein/4 h, respectively. Accordingly, the values of PEF- and MEF-treated cells were corrected for these effects. Values in each column with a different superscript letter are significantly different at $P < 0.05$.

TABLE 4

Inhibition of [1-^{14}C]Acetate Incorporation into Lipids of Hepatocytes by *S*-Allyl Cysteine and Kyolic[a]

Treatment	[1-^{14}C]Acetate incorporation into	
	Cholesterol	Fatty acid
	(% of control)[b]	
S-Allyl Cysteine (mM)		
0.05	98.4 ± 4.6[d]	99.6 ± 8.7[d]
0.1	91.7 ± 9.1[d–f]	96.8 ± 6.1[d]
0.5	112.2 ± 12.2[d,e]	95.4 ± 11.1[d]
1.0	115.7 ± 6.8[d]	90.1 ± 11.0[d]
2.0	79.3 ± 2.7[e–g]	90.1 ± 11.0[d]
4.0	73.4 ± 4.6[f,g]	83.6 ± 8.7[d]
Kyolic (mM)[c]		
0.01	102.8 ± 18.2[d]	84.9 ± 12.8[d]
0.05	77.5 ± 9.7[d,g]	128.4 ± 20.8[d,e]
0.1	27.5 ± 1.3[e,g]	155.5 ± 14.8[d,g]
0.2	24.2 ± 3.6[e–g]	122.1 ± 11.7[d]
0.4	13.2 ± 3.9[f,g]	28.9 ± 2.3[f,g]

[a]Hepatocytes were incubated in serum-free Dulbecco's modified Eagle medium containing 0.5 mM [1-^{14}C]acetate (specific activity, 1.0 mCi/mmol or 37 MBq/mmol).

[b]The mean specific activities of [1-^{14}C]acetate incorporation into cholesterol and fatty acid ranged from 121–158 and 610–825 pmol acetate/mg protein/4 h, respectively. The values for treatment groups are expressed as % of the control based on the mean specific activity determined from each of four experiments.

[c]Kyolic concentrations are expressed as *S*-allyl cysteine equivalents contained in the liquid garlic extract.

[d–f]Values are means ± SE for three experiments. The average of three wells measured in each experiment was considered a data point. Values in each column with a different superscript letter are significantly different at $P < 0.05$.

[g]Indicates a significant difference from the corresponding value of the control group.

TABLE 5

Effects of Garlic Extracts on the Rate of [2-^{3}H]Glycerol Incorporation into Glycerolipids of Hepatocytes[a]

Treatment[b]	[2-^{3}H]Glycerol + oleic acid		[2-^{3}H]Glycerol + acetic acid		
	Triacylglycerol	Phospholipids	Triacylglycerol	Diacylglycerol	Phospholipids
	(pmol glycerol incorporated/mg protein/4 h)				
Control	1076 ± 39[d]	352 ± 7[d]	1035 ± 27[c]	86 ± 1[c]	482 ± 9[c]
PEF	1157 ± 32[c,d]	349 ± 8[d]	895 ± 12[d]	68 ± 1[e]	357 ± 6[d]
MEF	1235 ± 26[c]	383 ± 8[c]	945 ± 32[d]	78 ± 1[d]	379 ± 13[d]
WEF	1174 ± 44[c,d]	452 ± 26[c]	906 ± 32[d]	69 ± 2[e]	382 ± 10[d]

[a]Hepatocytes were incubated in serum-free Dulbecco's modified Eagle medium containing 0.1 mM [2-^{3}H]glycerol (specific activity, 0.5 mCi/mmol or 18.5 MBq/mmol) and either 0.5 mM oleic acid complexed with bovine serum albumin or 1.0 mM sodium acetate. Garlic extracts equivalent to 1.25 mg of dry garlic powder in 5 μL was included in the incubation. When compared with the control group, addition of 5 μL ethanol or methanol decreased slightly (<2%), but not significantly, the rates of [2-^{3}H]glycerol incorporation into glycerolipids.

[b]PEF (petroleum ether), MEF (methanol) and WEF (water)-extractable fractions of fresh garlic were resuspended in ethanol, methanol and water, respectively.

[c–e]Values are means ± SE for three experiments. The average of three wells measured in each experiment was considered a data point. Values in each column with a different superscript letter are significantly different at $P < 0.05$.

0.4 mM *S*-allyl cysteine, Kyolic depressed the rate of synthesis by 87%. The rate of [1-^{14}C]acetate incorporation into fatty acids was inhibited by Kyolic at the high concentration (i.e., 0.4 mM equivalent), but was stimulated at 0.1 mM concentration.

Plasma TG levels depend in part on TG synthesis and secretion by the liver. TG synthesis, on the other hand, also competes with PL synthesis for substrates. Unlike the effects of the garlic extracts on cholesterol and fatty acid synthesis, none of the extracts inhibited the rate of [2-^{3}H]glycerol incorporation into TG when oleic acid was present in the culture medium (Table 5). The rate of incorporation into TG was increased 15% by MEF as compared to controls. Similarly, the rate of [2-^{3}H]glycerol incorporation into PL was not affected by PEF or MEF, but stimulated by 28% in the presence of WEF.

In a subsequent experiment, the rate of [2-^{3}H]glycerol incorporation into lipids was measured in the presence of 1.0 mM sodium acetate. As shown above, acetate is readily used by hepatocytes for fatty acid synthesis. Under our experimental conditions, the rates of [2-^{3}H]glycerol incorporation into TG were decreased 9–14% by PEF, MEF or WEF as compared to controls (Table 5). The rate of glycerol incorporation into diacylglycerol was lowered by all three garlic extracts. The garlic extracts were also effective in reducing the rate of glycerol incorporation into PL by 21–26%.

DISCUSSION

Garlic powder (7,10,12), garlic extracts (4,6,9,13,16–18), essential oils of garlic (12–14) and purified sulfur compounds from garlic (10,14) are known to reduce plasma of total cholesterol, low density lipoprotein-cholesterol and TG levels in animals and in humans. The present study on rats not only confirms these hypocholesterolemic and hypotriglyceridemic effects, but provides some additional insights into the mechanism(s) by which garlic lowers lipid levels in the circulation. Our data strongly suggest that the cholesterol-lowering action of garlic stems in part from its inhibition of hepatic cholesterol synthesis, which is consistent with conclusions drawn based on *in vitro* experiments with Hep G2 cells, a human hepatoma cell line and cultured rat hepatocytes (20). Maximal inhibition of cholesterogenesis, however, appears to require multiple components of garlic, as became evident through the following observations. First, PEF, MEF and WEF were all effective in depressing cholesterol synthesis, indicating that the active components of garlic are hydrophobic, or hydrophilic, or both. The inhibition of cholesterol synthesis coincided with a marked reduction in plasma total cholesterol levels by organic solvent and water-extractable garlic constituents fed to the animals (9,16–18). Second, the rate of [1-^{14}C]acetate incorporation into cholesterol was not altered by *S*-allyl cysteine at 0.05–1.0 mM and was decreased by only 20–30% in the presence of higher concentrations (i.e., 2.0 and 4.0 mM). Third, Kyolic, at a concentration equivalent to 0.04 mM *S*-allyl cysteine, inhibited hepatic cholesterogenesis to the same extent as did 2.0–4.0 mM purified *S*-allyl cysteine. A maximal inhibitory effect (i.e., 87%) was attained by Kyolic at a concentration equivalent to 0.4 mM *S*-allyl cysteine. A recent study by Sendl *et al.* (29) had shown that cholesterol synthesis *in vitro* could be markedly depressed by a variety of sulfur containing compounds from garlic (e.g., allicin, ajoene, diallyl disulfide) at 0.5–1.0 mM concentrations. In addition to *S*-allyl cysteine, Kyolic also contains other organosulfur compounds such as methyl sulfide, allyl disulfide and allylmethyl trisulfide (30). Thus, the potent inhibitory effects of Kyolic are likely to be due to the combined effects of these sulfur containing compounds.

The depressed rates of cholesterogenesis observed in the cultured hepatocytes may be related to the decreased activities of 3-hydroxy-3-methyl-glutaryl-CoA

reductase measured in the liver of animals fed diets supplemented with different garlic preparations (16–18). This notion is supported by the results of a recent study, that showed that water-soluble garlic extracts, when incubated with cultured hepatocytes, lowered the activity of the reductase (20), the rate-limiting enzyme early in cholesterol synthesis. Furthermore, when purified diallyl disulfide was preincubated with liver microsomes, the reductase was irreversibly inactivated (31). However, it should be pointed out that the garlic extracts could also inhibit steps later in the cholesterol metabolic pathway (20). For example, garlic extracts fed to chicken have been shown to decrease hepatic cholesterol 7α-hydroxylase activity (16,17). Furthermore, allicin has been shown to inhibit the activity of bovine acetyl CoA synthetase (32). Acetyl-CoA synthetase, in turn, was shown to exist both in the cytoplasm and in the mitochondria of rat liver (33). As [1-^{14}C]acetate was used as the substrate for cholesterol synthesis in the present study, it may be speculated that the decreased rate of [1-^{14}C]acetate incorporation into cholesterol induced by garlic extracts and Kyolic could involve inhibition of acetyl-CoA synthetase.

Likewise, the mechanism(s) underlying the hypotriglyceridemic effect of garlic is not completely understood. As hepatic TG production is an important factor in the regulation of plasma levels, the use of [2-^{3}H]glycerol for measuring the hepatic capacity for TG synthesis permitted us to ascertain a causal relationship between hepatic TG production and TG appearance in the circulation. The incorporation of [2-^{3}H]glycerol into TG is dependent upon the formation of *sn*-glycerol-3-phosphate and its esterification with fatty acids. Thus, any limitation in the availability of the glycerol backbone or of fatty acid could lead to a reduction in TG synthesis. In a series of experiments, we found that the rate of [2-^{3}H]glycerol incorporation into TG was significantly inhibited by PEF, MEF and WEF under conditions where acetate was provided as fatty acid precursor to the hepatocytes in the incubation medium. Such an inhibition was not observed when a fatty acid (i.e., oleic acid) was supplemented. Clearly, the TG-lowering effect of active garlic components must in part be attributed to a decreased rate of TG synthesis in the liver, which, in turn, is the result of depressed *de novo* fatty acid synthesis. Various garlic extracts fed to animals have been shown to decrease the activities of NADPH producing enzymes and of fatty acid synthetase (9,10,16–18). Therefore, it is likely that the impairment of fatty acid synthesis observed in the cultured cells stems partly from the inhibition of the lipogenic enzymes. The inhibition of TG production, however, does not seem to be due to competition with diacylglycerol or PL synthesis, which were similarly inhibited by the garlic extracts.

In summary, the cholesterol- and lipid-lowering effects of long-term treatment with garlic reported in various human intervention (2–6) and animal feeding studies (7–18) were assessed and confirmed here in short-term experiments using primary hepatocyte cultures. This cell culture model should prove useful for further screening of various garlic principles for their anticholesterogenic properties.

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Effect of Fish Oil on Cancer Cachexia and Host Liver Metabolism in Rats with Prostate Tumors

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The aim of this study was to investigate whether tumor-induced cachexia and aberrations in host liver metabolism, induced by the MAT-LyLu variant of the Dunning prostate tumor, could be prevented by ω3 fatty acids from fish oil. On day 0, adult Copenhagen-Fisher rats fed normal chow *ad libitum* were inoculated with 10^6 MAT-LyLu cells (n = 14) or saline (n = 9). On day 7, when tumors were palpable, four tumor-bearing (TB) and four nontumor-bearing (NTB) rats were put on isocaloric diets with 50% of total energy as fish oil. The introduction of fish oil-enriched diets caused a reduction in energy intake to less than half of the energy intake by animals fed normal diets during days 7–14 (difference by dietary group: NTB, $P < 0.001$; TB, $P < 0.001$). During days 14–21, energy intake in fish oil-fed animals returned to approximately 75% of energy intake by animals fed normal diets (difference by dietary group: NTB, $P < 0.003$; TB, $P = 0.001$). Carcass weight of animals on day 21, when the study was terminated, was significantly related to initial weight ($P = 0.05$) and mean food intake during the study ($P = 0.01$). When data were adjusted for these variables using analysis of covariance, with NTB animals on normal diets being the reference group, significant loss of carcass weight was observed in TB animals on normal diets only (mean ± SEM 58 ± 10 g loss, $P < 0.001$), but not in TB animals on fish oil diets (8 ± 18 g loss, $P = 0.67$). This positive effect of fish oil diets on carcass weight in TB animals was statistically significant (50 ± 19 g, $P < 0.02$), implicating that the fish oil enriched diet inhibited tumor-induced weight loss by more than 85%. No effect of fish oil diets on tumor growth was detected. In all TB animals, regardless of diet, hepatic [P_i]/[adenosine triphosphate] ratios measured by ^{31}P magnetic resonance spectroscopy (MRS) *in vivo* and *in vitro* were elevated, and absolute concentrations of phosphocholine, glycerophosphocho-line, glycerophosphoethanolamine and glucose-6-phosphate as determined by ^{31}P MRS *in vitro* were reduced. Ultrastructural studies of liver tissue revealed increased numbers of mitochondria and increased amounts of endoplasmic reticulum in the host liver of TB animals, without differences between dietary group. In conclusion, fish oil supplementation partially inhibited MAT-LyLu tumor-induced cachexia, but did not prevent the majority of ^{31}P MRS-detectable alterations in host liver metabolism.

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There is increasing evidence suggesting that ω3 fatty acids from fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may inhibit carcinogenesis and tumor growth (1–8). In cultures of human cancer cell lines, EPA, but not DHA, was cytotoxic to tumor cells to a greater extent than to nontumorigenic cells (3). In a number of chemically-induced and transplanted tumors, fish oil reduced tumor incidence, delayed tumor appearance, and decreased the growth rate and size of tumors (4–7). In a cachexia-inducing rodent tumor, fish oil given after tumors were palpable, inhibited tumor growth and carcass weight loss in a dose-dependent fashion (8). Different possible mechanisms underlying these effects, such as modulation of oncogene expression, cell-mediated immune response, prostaglandin synthesis and lipid metabolism, have been discussed (1–8).

We have recently reported (9) that the MAT-LyLu variant of the Dunning prostrate tumor, when transplanted into Copenhagen-Fisher rats, induces weight loss and marked alterations in host liver metabolism despite lack of metastatic involvement in the liver, and without a concomitant reduction in food intake. These alterations in the host liver included a rise in [P_i]/adenosine triphosphate (ATP)] ratios and a marked reduction in glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) concentrations (9). In contrast, fish oil supplementation in healthy animals significantly increased hepatic GPC levels (10). Based on these findings and previous reports on anticachectic effects of fish oil (8), the present study was aimed at investigating the effects of fish oil supplementation on weight loss and host liver metabolism in rats bearing MAT-LyLu tumor allografts.

MATERIALS AND METHODS

Animals. Fourteen 12-week-old male Copenhagen-Fisher F1 hybrid rats (Harlan-Olac Ltd., Bicester, Oxford, United Kingdom) were used for this study. Mean (± SEM) weight of the animals was 321 ± 2 g. All animals were treated identically throughout the study, unless stated otherwise.

Tumor inoculation. MAT-LyLu rat prostate carcinoma cells were obtained from Dr. J. Isaacs (Johns Hopkins Cancer Center, Baltimore, MD) and cultured as previously described (11). At confluence, cells were harvested using 0.025% trypsin in 10 mM sodium phosphate buffer (pH 7.4) without Ca^{2+} or Mg^{2+}. Animals were inoculated into subcutaneous tissues of the left flank with 1×10^6 tumor cells suspended in 500 μL of sterile saline. Nine animals were injected with saline as controls.

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F6P, fructose-6-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; MRS, magnetic resonance spectroscopy; NTB, nontumor-bearing; *sn*-GP, *sn*-glycerol-3-phosphate; TB, tumor-bearing; TR, repetition time.

Diet and body weight. After tumor inoculation, animals were maintained in adjacent individual cages with water and standard food available *ad libitum*. The standard food was rat and mouse breeding diet (Pilsbury's Ltd., Birmingham, United Kingdom), which contained 50% carbohydrate, 20% protein, and 4.1% crude oil and supplied 11.5% of the energy as fat. Essential fatty acids comprised 1.45% of the diet (8). Seven days after tumor inoculation, when tumors were just palpable in all tumor-bearing (TB) animals, four TB and four nontumor-bearing (NTB) rats were changed onto a diet containing 50% of total energy as fish oil. The fish-oil enriched diet was prepared as an isocaloric isonitrogenous diet by decreasing the carbohydrate content and supplying the remaining energy from MaxEPA fish oil (kindly supplied by Seven Seas Ltd., Hull, United Kingdom) (8). The peroxide value (12) of fish oil was 4.6 mg/kg, which is within the quality specification for vacuum deodorized fish oil from the National Oceanic and Atmospheric Administration of the United States (13). Food was prepared fresh daily throughout the study. Body weight, as well as food and water intake, was monitored daily. Carcass weight was calculated by subtracting the weight of primary and metastatic tumors from total body weight.

Anesthesia. At 21 d after tumor inoculation, animals were fasted overnight and anesthetized using 0.5 mL/kg Hypnorm^{R} (Janssen Pharmaceutical Ltd., Grove, Oxford, United Kingdom), containing 0.315 mg fentanyl citrate and 10 mg fluanisone per mL, and 1 mg/kg Hypnovel (midezolam; Roche Laboratories, Division of Hoffmann-La Roche Inc., Nutley, NJ). A midline laparotomy was performed, and a two-turn, 14-mm diameter coil was placed immediately over the liver with an aseptic layer of film placed between the coil and the exposed tissue. Animals were maintained at 37°C throughout the experiment *via* a heated pad.

Magnetic resonance spectroscopy (MRS) in vivo. MRS data were acquired *in vivo* using an SIS MR imaging spectrometer (Spectroscopy Imaging Systems Corporation, Sunnyvale, CA) interfaced to an Oxford 4.7 Tesla, 30-cm bore superconducting magnet (Oxford Instruments Ltd. Eynsham, Oxford, United Kingdom). In order to optimize magnetic field homogeneity in the region of the coil, the coil was transmission line tuned to 200.06 MHz for detection of ^{1}H MRS signals. Typical linewidths of 50 Hz were observed for the water resonance. ^{31}P MRS data were then acquired with the coil tuned to 80.98 MHz, using a pulse of approximately 45° at the center of the coil, and a repetition time (TR) of 8 s (collecting 64 signal averages). Spectral quantification was carried out identically for all ^{31}P MRS data, in a blinded fashion. First, baseline correction was performed as follows: for each data set, Fourier transformation was carried out twice, applying line broadening of 20 Hz and 500 Hz, respectively. The two resulting spectra were then subtracted to obtain a spectrum corrected for the broad phospholipid signal (14). Resonances of interest were identified by chemical shift and quantitated by manual integration. For peaks which were not completely resolved, (i.e. phosphomonoesters, P_i and phosphodiesters), peak areas were defined as previously described (15). It was found empirically that use of TR 8 s and a pulse angle of 45° allowed for adequate relaxation of all resonances of interest obtained on the liver, i.e., use of a longer TR did not make a significant difference to the relative ratios of the measured resonance intensities. Hence, no correction for saturation was necessary.

MRS in vitro. On completion of MRS *in vivo*, part of the liver was freeze-clamped *in situ* at liquid nitrogen temperature (16). Tissue samples were extracted with perchloric acid (17), and adjusted to pH 7.5 using potassium hydroxide. The resulting supernatant was lyophilized and redissolved in D_2O. After adding ethylenediaminetetraacetic acid to a final concentration of *ca.* 100 mM, pH was readjusted to 7.5. High resolution ^{31}P MRS spectra were acquired on a 8.4 Tesla Bruker system (Bruker Spectrospin Ltd., Coventry, United Kingdom), using a 35° excitation pulse with TR 10 s. Proton scalar coupling interactions were removed by using low power proton decoupling. Metabolites *in vitro* were identified in a related study (17) by a combination of chemical shift, pH titration and standard addition of known compounds. A peak was considered identified if all these criteria were fulfilled. Concentrations *in vitro* were corrected for saturation and nuclear Overhauser effects by obtaining spectra at long TR (20 s) and by limiting the use of low power proton decoupling to the period of spectral acquisition only. Absolute concentrations of metabolites were obtained by calculating the peak area of each metabolite relative to the peak area of a standard metabolite of known chemical shift and concentration (phosphocreatine), which was added to the medium.

Microscopy. For light microscopy, specimens were fixed in fresh, neutral-buffered formaldehyde (10% vol/vol). After routine embedding in paraffin wax, tissue sections were cut at 3 μM thickness with a microtome and stained with hematoxylin and eosin. For electron microscopy, 1-mm cubes of tissue were fixed in 2% glutaraldehyde buffered with 0.2 M sodium phosphate-buffer (pH 7.2). Tissue blocks were post fixed in 1% osmium tetroxide in Millonig's buffer (18) for 1 h at room temperature. Thereafter, following two washes for 30 min each in distilled water, blocks were dehydrated in graded alcohols followed by three changes of TAAB^{R} resin (TAAB Laboratories, Aldermaston, United Kingdom) for one hour each. Tissue blocks were embedded in polyethylene capsules (BEEM capsules; TAAB Laboratories) in fresh identical resin. Polymerization was performed at 60°C overnight. Blocks were cut at 1 mm on a Reichert ultranicrotome using a glass knife. Serial ultrathin sections were then cut at 80 nm thickness using a diamond knife. Sections were placed onto uncoated nickel grids and stained in saturated methanolic uranyl acetate for 3 min followed by Reynold's lead citrate (19) for 8 min, both at room temperature. After drying, sections were examined and photographed using a Philips CM10 electron microscope (Philips Export BV, Eindhoven, The Netherlands) at 80 kV.

Statistical analysis. Results as expressed as means ± SEM in μmol per g wet weight and analyzed for significance by Student's *t*-test for independent groups; for

concentrations on P_i and P_i/ATP ratios (which were not distributed normally), Wilcoxon's rank sum test was used. Multivariate models were fitted in order to investigate whether the effect of fish oil diets on body weight gain during days 7–14 and days 14–21 was different in NTB and TB animals, including as covariates dummy variables for: (i) TB state; (ii) dietary group; and (iii) interaction of TB state and diet. In order to determine the effect of TB state and diet on changes in carcass weight after adjustment for food intake, analysis of covariance was used, including, as covariates: initial weight, mean daily food intake during the study, and dummy variables for: (i) TB state and (ii) interaction between TB state and diet. P values less than 0.05 were considered statistically significant.

RESULTS

Tumor weight. No effect of fish oil diets on primary or metastatic tumor weights was detected. Primary tumor weight on day 21 after tumor inoculation, when the study was terminated, was 67 ± 6 g (mean ± SEM) in TB animals on normal diets and 67 ± 8 g in animals on fish oil diets ($P > 0.10$). Metastatic tumor weight on day 21 was 9 ± 2 g in TB animals on normal diets and 7 ± 4 g in TB animals on fish oil diets ($P > 0.10$).

Food intake, total body and carcass weight. Food intake in NTB animals on normal diets (control group) was constant during the first two weeks of the study, but decreased during the third week (Fig. 1; $P < 0.002$, as compared to week 2). This decrease in food consumption was paralleled by attenuation of growth during the third week of the study (Fig. 2; days 18–21 as compared to days 14–18, $P < 0.002$), reflecting the animals reaching adult age (i.e., 14–15 wk of age).

Food intake during the first week of the study, when normal diets were given to all animals, was not significantly different between any of the NTB and TB groups ($P > 0.10$). However, the introduction of fish oil-enriched diets in part of NTB and TB animals on day 7 caused a reduction in food and energy intake to less than half of the energy intake by animals on normal diets during the second week of the study (difference by dietary group: NTB, $P < 0.001$; TB, $P < 0.001$). In the third week of the study, food intake in fish oil-fed animals returned to levels of approximately 75% of those in animals on normal

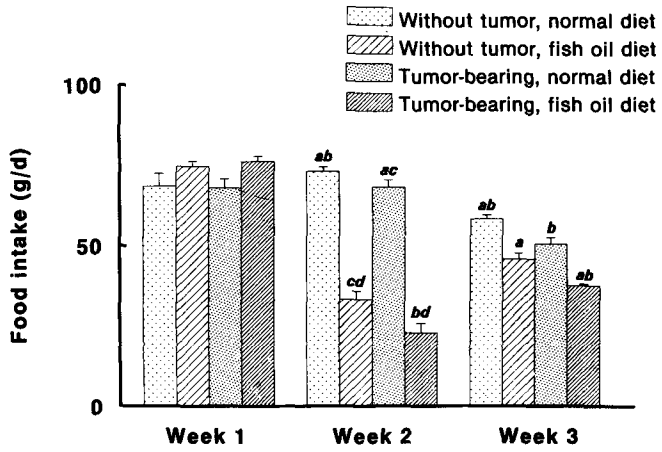

FIG. 1. Food intake by healthy rats and rats with tumors, on different diets. Boxes show means and bars SEM; values within one period with the same letter are significantly different (Students' *t*-test, $P < 0.05$). From days 0–7 (week 1), all animals received standard rat chow *ad libitum*. From day 7 onward (weeks 2 and 3), part of the animals were given a diet with 50% of energy from fish oil. As seen in the figure, the introduction of fish oil diets induced a sharp temporary reduction in food intake in fish oil-fed animals during the second week of the study. In the third week, food intake of these animals returned to levels close to those in animals fed normal diets.

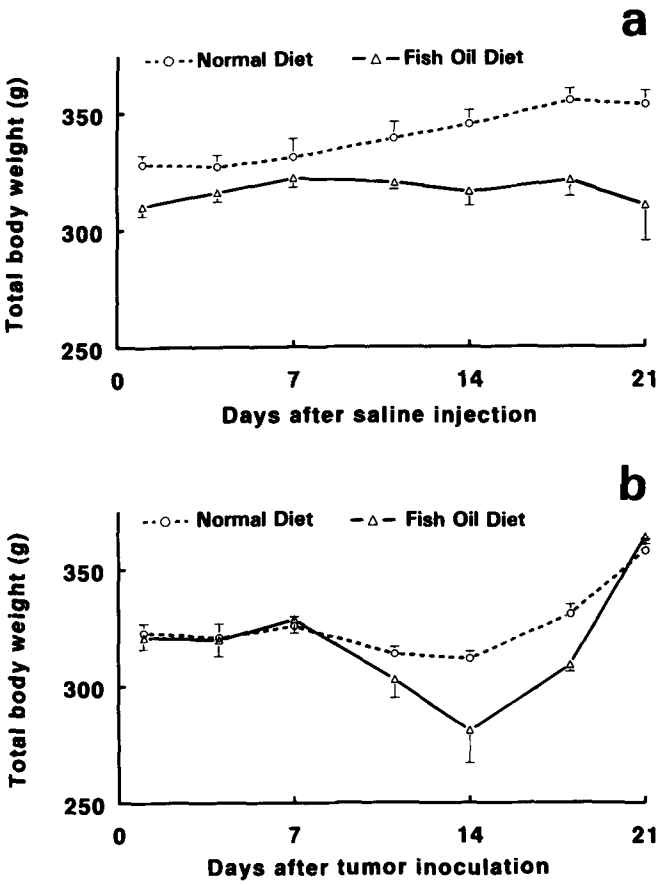

FIG. 2. Total body weight of (a) nontumor-bearing (NTB) rats and (b) tumor-bearing (TB) rats, on different diets. Fish oil-enriched diets were given from day 7 onward. Between-group differences in weight gain: days 1–7, no significant differences; days 7–14, a relative weight reduction is observed in NTB and TB animals on fish oil diets when compared to NTB and TB animals on normal diets, respectively (NTB, $P = 0.03$; TB, $P < 0.005$), with no significant interaction between the effects of diet and TB state (detected by multivariate analysis as described in the Materials and Methods section, $P > 0.10$). From days 14–21, weight gain in NTB animals fed fish oil diets was still depressed ($P > 0.10$ as compared to NTB animals on normal diets), whereas TB animals fed fish oil diets showed accelerated weight gain as compared to TB animals fed normal diets ($P < 0.02$). This positive effect of fish oil diets on weight gain in TB animals was significantly different from the effect of diet on weight gain in NTB animals (multivariate analysis, $P = 0.03$).

diets (Fig. 1), but differences by dietary group were still statistically significant (NTB, $P < 0.003$; TB, $P = 0.001$).

Changes in total body weight of animals during the study are shown in Figure 2a (NTB animals) and Figure 2b (TB animals). In the first week of the study, when all animals received normal diets, no significant differences in body weight or in weight gain were observed between any of the NTB or TB groups. During the second week (days 7–14), TB animals on normal diets lost weight relative to NTB (control) animals on normal diets (Fig. 2; $P < 0.001$). Over the same period, NTB and TB animals on fish oil diets showed significant weight loss when compared with NTB and TB animals on normal diets, respectively (Fig. 2, difference by dietary group: NTB, $P = 0.03$; TB, $P < 0.005$). This effect of dietary group was not significantly different between NTB and TB animals ($P > 0.10$). During the third week of the study (days 14–21), weight gain in NTB animals on fish oil diets remained depressive relative to NTB animals on normal diets, even though the difference no longer reached statistical significance (Fig. 2a; $P > 0.10$). In contrast, TB animals on fish oil diets showed a marked catch-up in body weight during the same period (Fig. 2b; $P < 0.02$, as compared to TB animals on normal diets). This positive effect of fish oil diets on body weight in TB animals was significantly different from that in NTB animals ($P = 0.03$). Because, as already described, final tumor weight was similar in TB animals irrespective of diet, the effect of fish oil diets on body weight of TB animals was attributed to changes in carcass weight.

Effect of food intake on carcass weight. The effect of fish oil enriched diets on final carcass weight on day 21, when the study was terminated, was further analyzed using analysis of covariance. In this model, initial weight and food intake over the three-week study period were included as covariates because they were significantly related with carcass weight on day 21 (for initial weight, $P = 0.05$; for food intake, $P = 0.01$). Taking NTB animals on normal diets as the reference group, significant loss of carcass weight was demonstrated in TB animals on normal diets only (58 ± 10 g loss, $P < 0.001$), but not in TB animals on fish oil diets (8 ± 18 g loss, $P = 0.67$). This positive effect of fish oil diets on carcass weight in TB animals was statistically significant (50 ± 19 g, $P < 0.02$), implicating that fish oil diets inhibited tumor-induced weight loss by more than 85%.

Liver metabolism. We previously reported (9) a rise in $[P_i]$/[ATP] ratios in TB animals on normal diets when compared to NTB animals on normal diets. Similarly elevated $[P_i]$/ATP] ratios were observed in TB animals on fish oil diets (Fig. 3 and Table 1). No significant differences in [phosphomonoester]/[ATP] ratios were observed between any of the groups. [Phosphodiester]/[ATP] ratios were reduced in TB animals on fish oil diets when

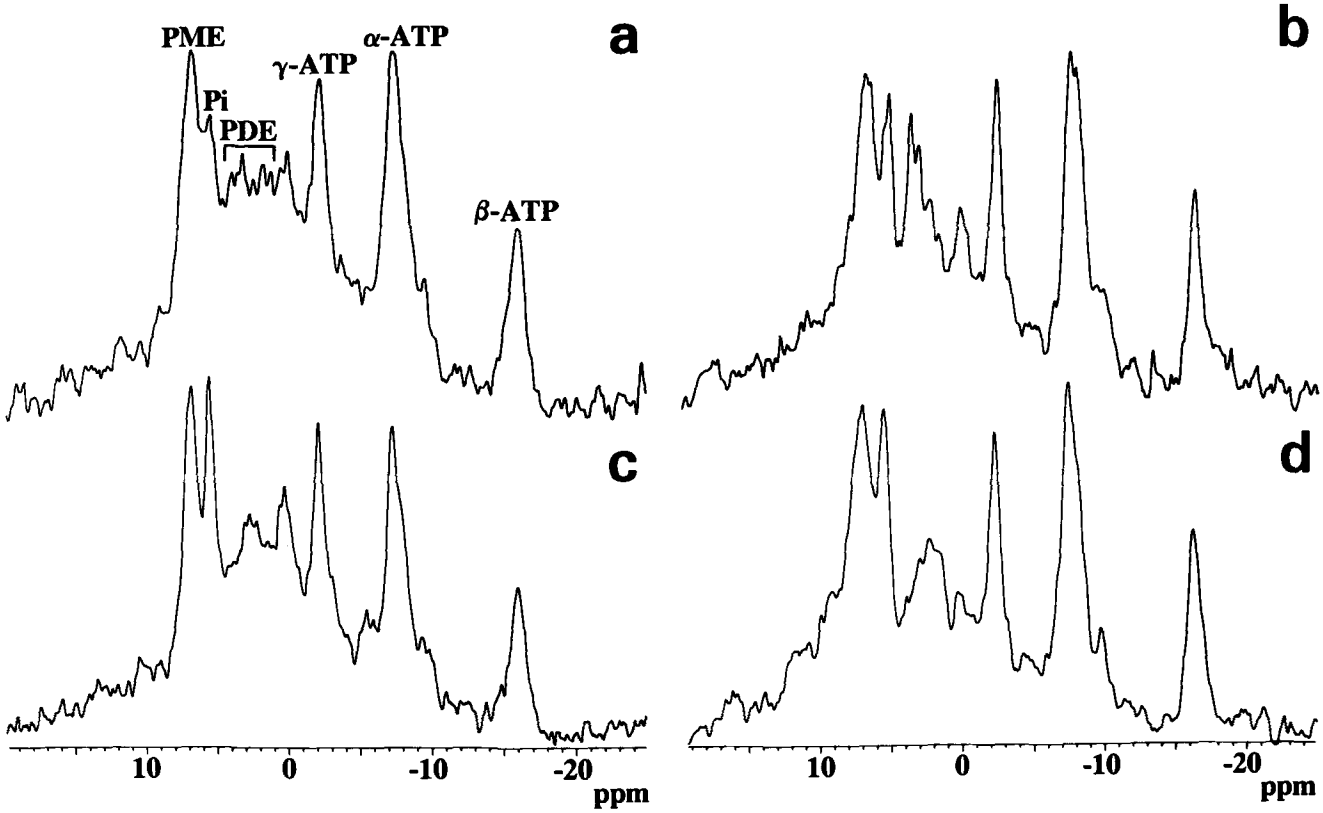

FIG. 3. ^{31}P magnetic resonance spectroscopy spectra *in vivo* of the liver from healthy rats and rats with tumors, on different diets: (a) animals without tumors and taking normal diets; (b) animals without tumors taking fish oil-enriched diets; (c) tumor-bearing animals on normal diets; and (d) tumor-bearing animals on fish oil-enriched diets. MRS spectra were obtained at 4.7 Tesla (45° acquisition pulse, repetition time 8 s) as described in the Materials and Methods section. Peak assignments: α-ATP, β-ATP, γ-ATP: α-, β- and γ-phosphate groups of adenosine triphosphate. PDE, phosphodiesters; PME, phosphomonoesters.

compared with TB animals on normal diets ($P < 0.005$), and with NTB animals on either diet ($P < 0.05$). No significant difference in intracellular pH was observed between any of the groups.

^{31}P MRS of liver extracts (Fig. 4 and Table 2) confirmed the elevated $[P_i]/[ATP]$ ratios observed *in vivo* in TB animals of either dietary group. Furthermore, absolute adenosine monophosphate concentrations in the liver of TB animals on fish oil diets were more than twice the levels in the other three groups. No difference in adenosine diphosphate concentrations was observed between any of the groups. Glucose-6-phosphate (G6P) concentrations were reduced in all TB animals regardless of diet, even though the difference from NTB animals reached statistical significance for TB animals on normal diets only ($P < 0.04$). In contrast, fructose-6-phosphate (F6P) concentrations were significantly higher in TB animals on fish oil diets ($P = 0.001$) than in TB animals on normal diets, reaching values close to NTB animals on normal diets. Concentrations of *sn*-glycerol-3-phosphate (*sn*-GP) were significantly higher in TB animals on fish oil diets when compared with the other groups ($P \leq 0.02$, Table 2). Glyceraldehyde-3-phosphate (G3P) levels were also increased, but this difference was only significant when compared with NTB animals on fish oil diets. No differences in 3-phosphoglycerate concentrations were observed. Phosphocholine, GPC and GPE concentrations were significantly decreased in the liver of all TB animals regardless of diet (Fig. 5).

Histological and ultrastructural studies of liver tissue. Microscopic examination revealed no region of congestion of ischemia within the liver of any of the animals. Histological examination confirmed the absence of metastatic tumor cells from the host liver of all TB animals. Microscopic appearance of the liver was normal,

TABLE 1

Metabolite Ratios in the Liver of Rats Without Tumors and Tumor-Bearing Rats Fed Normal or Fish Oil-Enriched Diets as Determined by Magnetic Resonance Spectroscopy *in vivo*[a,b]

	Nontumor-bearing		Tumor-bearing	
	Normal diet (n = 5)	Fish oil diet (n = 4)	Normal diet (n = 6)	Fish oil diet (n = 4)
$[P_i]/[ATP]$	$0.78 \pm 0.10^{c,d}$	$0.83 \pm 0.11^{e,f}$	$2.04 \pm 0.37^{c,e}$	$1.64 \pm 0.29^{d,f}$
[PME]/[ATP]	1.91 ± 0.31	1.78 ± 0.23	1.55 ± 0.17	2.23 ± 0.39
[PDE]/[ATP]	1.26 ± 0.08^{c}	1.56 ± 0.15^{d}	1.69 ± 0.15^{c}	$0.81 \pm 0.14^{c,d}$

[a]On day 0, Copenhagen-Fisher rats were inoculated with MAT-LyLu tumor cells (tumor-bearing animals), or saline (nontumor-bearing controls). From day 7 onward, some of the nontumor-bearing and tumor-bearing animals were anesthetized, and ^{31}P MRS spectra were obtained at 4.7 Tesla (45° excitation pulse, repetition time 8 s) as explained in the Materials and Methods section.
[b]Abbreviations: PME, phosphomonoesters; PDE, phosphodiesters; MRS, magnetic resonance spectroscopy; ATP, adenosine triphosphate.
[c–f]Values shown are means ± SEM. Values in a row with the same superscript letter are significantly different ($P < 0.05$, using Student's *t*-test, except for $[P_i]/[ATP]$, where Wilcoxon's rank sum test was used).

TABLE 2

Metabolite Concentrations in the Liver of Rats Without Tumors and Tumor-Bearing Rats Fed Normal or Fish Oil-Enriched Diets, as Determined by ^{31}P Magnetic Resonance Spectroscopy *in vitro*[a,b]

	Nontumor-bearing		Tumor-bearing	
	Normal diet (n = 5)	Fish oil diet (n = 4)	Normal diet (n = 6)	Fish oil diet (n = 4)
Absolute concentrations				
ATP	$3.53 \pm 0.29^{c,d}$	2.05 ± 0.48^{c}	2.55 ± 0.40	2.36 ± 0.27^{d}
ADP	1.54 ± 0.28	1.12 ± 0.24	1.37 ± 0.24	1.71 ± 0.05
AMP	0.32 ± 0.07^{c}	0.36 ± 0.05^{d}	0.47 ± 0.12^{e}	$1.00 \pm 0.12^{c-e}$
P_i	8.43 ± 1.90^{c}	$3.88 \pm 0.58^{c-e}$	11.13 ± 2.40^{d}	10.83 ± 1.12^{e}
G6P	0.32 ± 0.08^{c}	0.16 ± 0.05	0.12 ± 0.04^{c}	0.12 ± 0.07
F6P	0.64 ± 0.22^{c}	0.27 ± 0.08	$0.17 \pm 0.04^{c,d}$	0.43 ± 0.02^{d}
sn-GP	0.15 ± 0.04^{c}	0.18 ± 0.04^{d}	0.16 ± 0.02^{e}	$0.42 \pm 0.07^{c-e}$
G3P	0.24 ± 0.13	0.15 ± 0.05^{c}	0.30 ± 0.10	0.52 ± 0.10^{c}
3PGA	0.48 ± 0.14	0.27 ± 0.13	0.29 ± 0.06	0.34 ± 0.05
Relative concentration				
$[P_i/ATP]$	$2.32 \pm 0.41^{c,d}$	2.70 ± 1.23	4.93 ± 1.20^{c}	4.80 ± 0.79^{d}

[a]Animal preparation as in Table 1. Liver was freeze-clamped *in situ,* and ^{31}P MRS spectra of tissue extracts were acquired at 8.4 Tesla, as described in the Materials and Methods section.
[b]Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; 3PGA, 3-phosphoglycerate; *sn*-GP, *sn*-glycerol-3-phosphate; MRS, magnetic resonance spectroscopy.
[c–e]Values shown are means ± SEM in μmol/g wet tissue. Values in a row with the same superscript letter are significantly different ($P < 0.05$, using Student's *t*-test, except for P_i and $[P_i]/[ATP]$, where Wilcoxon's rank sum test was used). Control values from tumor-bearing and nontumor-bearing animals on normal diets were taken from Reference 9, with permission.

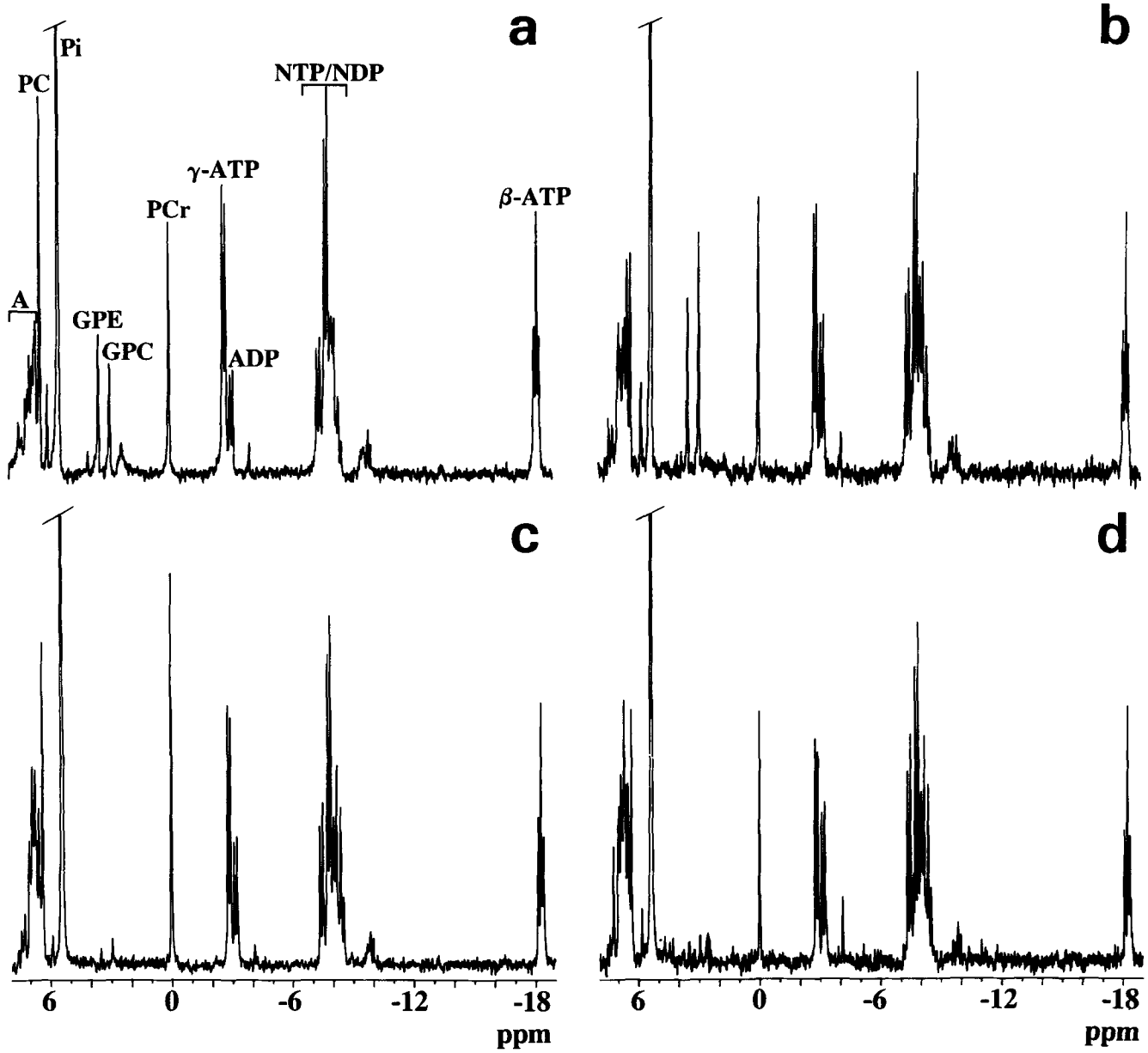

FIG. 4. ^{31}P MRS spectra of tissue extract in *in vitro* from liver of healthy rats and rats with tumors, on different diets: (a) animals without tumors and taking normal diets; (b) animals without tumors taking fish oil-enriched diets; (c) tumor-bearing animals on normal diets; and (d) tumor-bearing animals on fish oil-enriched diets. MRS spectra were obtained at 8.4 Tesla as described in the Materials and Methods section. The scale of the four spectra shown has been standardized to equal peak heights of β-ATP. Peak assignments: A, phosphomonoesters, including adenosine monophosphate, sugar phosphates and phosphocholine; β-ATP, γ-ATP: β- and γ-phosphate groups of adenosine triphosphate, G6P, glucose-6-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; NTP/NDP, nucleotide triphosphates and diphosphates; PC, phosphocholine; PCr, phosphocreatine (added as an internal chemical shift reference and quantitative standard).

without any inflammatory cell infiltration. Ultrastructural studies of liver tissue revealed the presence of small amounts of retained intracytoplasmic lipid within the cytoplasm of hepatocytes from NTB animals fed fish oil diets (Fig. 6). In all other respects, no differences were observed in the ultrastructural appearances of the NTB groups. However, there were distinct differences between NTB and TB animals. In both TB groups, mitochondria were increased in number. Typically, they were enlarged, swollen and irregular in appearance. In both groups of TB animals, the endoplasmic reticulum was increased in amount and in distribution throughout the hepatocyte cytoplasm. Individual cisternae of the rough endoplasmic reticulum were longer and more convoluted than those of the NTB groups. Generally, a much closer apposition of the cisternae to mitochondria was observed in hepatocytes from TB animals when compared to NTB animals. In contrast to the NTB animals fed on

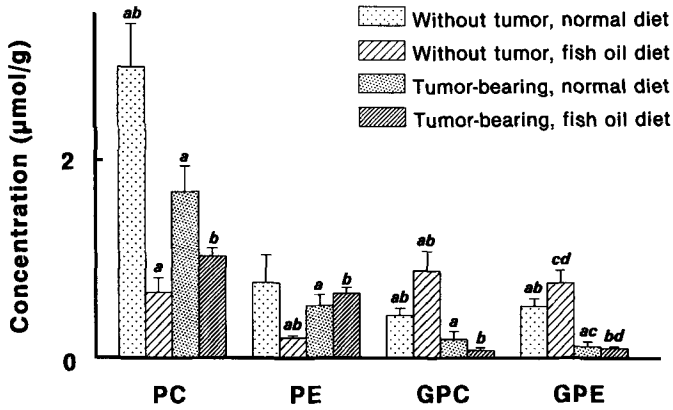

FIG. 5. Absolute concentrations of intermediates of phospholipid metabolism in the liver of healthy rats and rats with tumors, on normal or fish oil-enriched diets. Concentrations were measured using ^{31}P MRS *in vitro*. Abbreviations as in Figure 3, and PE, phosphoethanolamine. Boxes show means and bars SEM in µmol/g wet tissue. Values of one metabolite with the same letter are significantly different (Student's *t*-test, $P < 0.05$).

fish oil diets, hepatocytes of TB rats on fish oil diets did not contain cytoplasmic lipid droplets.

DISCUSSION

The aim of this study was to determine whether a diet rich in ω3 fatty acids from fish oil could reverse tumor-induced cachexia and changes in hepatic metabolism reported previously (9) in the host liver of rats with malignant prostate tumors. For this purpose, the MAT-LyLu variant of the Dunning prostate tumor was chosen as a cachexia model for two reasons. First, MAT-LyLu cells do not metastatize to the liver, as was confirmed by the absence of metastatic tumor cells at histological and electron microscopic examination of liver tissue. Second, as previously reported (9), loss of carcass weight in animals with MAT-LyLu tumors fed normal diets occurs without a significant reduction in food intake, suggesting that the cachexia induced by this tumor model is due to metabolic abnormalities in the host.

The introduction of a diet containing 50% of energy as fish oil, seven days after tumor/saline inoculation, caused a marked temporary reduction in food intake during the second week of the study, regardless of whether or not rats had tumors. Although food intake recovered to almost normal levels in the third week of the study, the temporary reduction was unexpected in the view of earlier reports, where an identically prepared fish oil diet did not lead to any decrease in food intake by NMRI mice with MAC16 colon carcinomas (8, 20). The cause of this reduced food intake in our study remains uncertain. Peroxidation of fish oil diets is unlikely to have played a significant role, as the fish oil used contained low peroxide levels and diets were freshly prepared every day throughout the study. The observation that in the third week of the study, food intake in fish oil fed animals returned to a level close to that in animals on normal diets (Fig. 1), would suggest that the animals initially did not find the fish oil diet palatable, and had to get used to this diet.

Nevertheless, when data were adjusted for differences in food intake, a significant inhibition of tumor-induced loss of carcass weight by more than 85% over the three-week study period was demonstrated in TB animals on fish oil diets when compared with TB animals on normal diets. It is noteworthy that TB animals fed fish oil diets showed a marked catch-up in body weight during the third week of the study (Fig. 2b), when their food intake was almost normal (i.e., at the same level as in TB animals fed normal diets), whereas no positive effects of fish oil diets on body weight were detected during the second week of the study, when the animals' intake of fish oil diets was low. This observation further supports a specific inhibition of weight loss in TB animals by fish oil diets. Our findings confirm earlier reports by Tisdale and co-workers (8), who found that a diet containing 50% of energy as fish oil, or the equivalent dose of pure EPA (21), completely prevented weight loss in mice with MAC16 tumors.

In contrast with the observations in MAC16 TB mice (8), fish oil diets did not inhibit the growth of MAT-LyLu tumors in this study. The possibility that this was due to the temporary reduction of food intake in animals on fish oil diets appears unlikely, as caloric restriction was previously reported to potentiate the inhibition of tumor growth by fish oil in experimental carcinomas (2). Therefore, the lack of inhibition of growth of the MAT-LyLu tumor is more likely to be related to kinetic properties of this tumor when compared with the MAC16 tumor. The latter is a slowly growing tumor producing cachexia at an early stage, when tumor mass is less than 1% of body weight (20,22), whereas the MAT-LyLu tumor is a rapidly growing tumor. This highly aggressive tumor may not allow for any inhibition of tumor growth by relatively mild therapeutic modalities, such as fish oil.

Fish oil diets did not prevent the majority of tumor-induced alterations in host liver metabolism as measured by ^{31}P MRS. A dramatic reduction in hepatic phosphorylation status and in concentrations of phosphocholine, GPC, GPE and G6P was observed in all TB animals, regardless of diet. The reduction in GPC levels in TB animals on fish oil diets was in marked contrast with the previously reported increase in GPC levels in NTB animals on fish oil diets (10). Our data suggest that a fish oil-enriched diet is not able to prevent these major ^{31}P MRS-detectable disturbances in host liver metabolism of animals with MAT-LyLu tumors, even though the rise in F6P concentrations in fish oil-fed animals would be compatible with an effect of ω3 fatty acids on the glycolytic/gluconeogenic pathway within the liver.

Ultrastructural examination of hepatocytes from all four groups of animals within this study demonstrated the presence of free cytoplasmic lipid droplets in hepatocytes from NTB animals on fish oil diets only. Triacylglycerol accumulation in the liver was previously reported in healthy rats on diets containing 30% of energy as fish oil and attributed to inhibition of triacylglycerol secretion by fish oil (23). In contrast, we did not detect

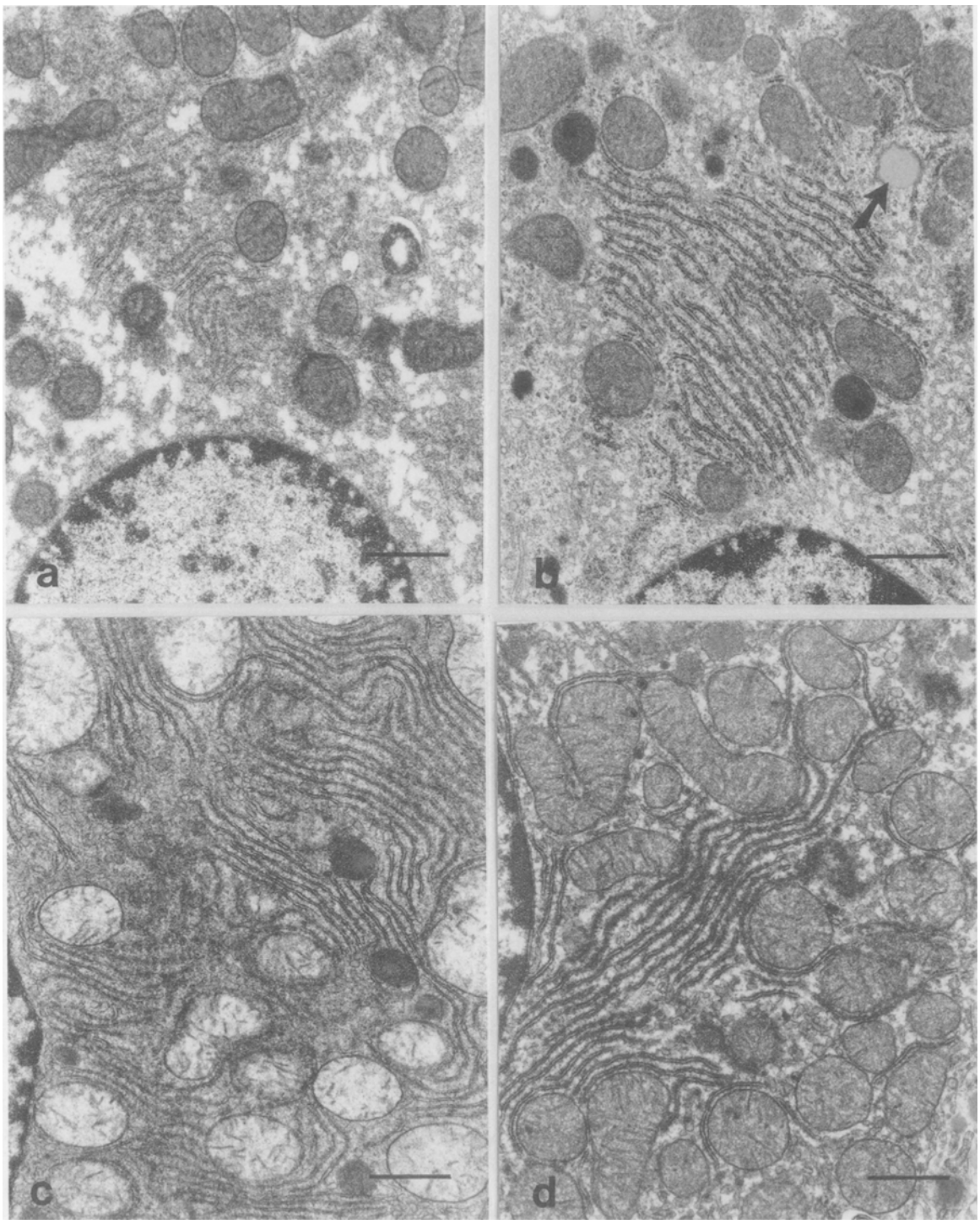

FIG. 6. Ultrastructural appearances of hepatocytes from healthy rats and rats with tumors, on different diets: (a) animals without tumors and taking normal diets; (b) animals without tumors taking fish oil-enriched diets; (c) tumor-bearing animals on normal diets; and (d) tumor-bearing animals on fish oil-enriched diets. Liver tissue was removed at 21 d after tumor inoculation (controls: saline injection) and fixed as described in the Materials and Methods section. In all control livers, mitochondria appeared small, uniform and compact. The endoplasmic reticulum was concentrated into well-localized regions adjacent to the nuclei. Hepatocytes from control rats fed high-fat diets appeared similar, but also contained lipid droplets (arrow). Hepatocytes from both groups of tumor-bearing animals contained increased numbers of "open," enlarged and irregularly-shaped mitochondria. The endoplasmic reticulum was increased in amount and in distribution throughout the cytoplasm. Cytoplasmic lipid droplets were not a characteristic feature of tumor-bearing animals fed high fat diets. In each electron micrograph, the bar indicates 1 μm.

any formation of lipid droplets in hepatocytes in TB animals on fish oil diets. This could be due to either decreased fatty acid precursor supply to the liver, to kinetic alterations within the liver or to increased peripheral utilization of triacylglycerols. The presence of a circulatory lipid mobilizing factor has been demonstrated in blood from mice with MAC16 tumors (21) and in cancer patients (22). EPA inhibited tumor-induced lipolysis from adipose tissue, leading to a reduction in plasma free fatty acid levels (21) and, thus, to decreased fatty acid supply to the liver. We recently reported (24) a rapid and marked reduction in free fatty acid concentrations in the blood of healthy humans after fish oil supplementation, suggesting that inhibition of lipolysis by fish oil may be a general phenomenon that is not specific to the TB state. Therefore, it is unlikely that reduced lipolysis would be the sole explanation of the absence of lipid droplets from the liver of TB animals. An alternative explanation is that fish oil may induce specific kinetic alterations within the liver of TB animals. The observation that, only in TB animals, fish oil diets induced a rise in *sn*-GP levels (Table 2) draws attention to the possible inhibition of an early step in glycerolipid synthesis by ω3 fatty acids (25). The simultaneous elevation of G3P (Table 2) would appear to exclude an alternative explanation for rising *sn*-GP, i.e., altered redox status of hepatocytes.

Generally speaking, however, the major differences in ultrastructure of hepatocytes in this study were observed between animals in the TB vs. NTB state, irrespective of diet. TB animals showed appearances similar to those previously reported (9), suggesting that the main influence on morphology was not the effect of diet, but of the tumors.

In conclusion, this study has shown that a diet containing 50% of total energy as fish oil significantly inhibited tumor-induced weight loss in rats with MAT-LyLu tumors. However, the fish oil-enriched diet did not reverse the majority of ^{31}P MRS-visible metabolic aberrations or ultrastructural abnormalities in the host liver of these rats. Furthermore, no effect of fish oil on the growth rate of MAT-LyLu tumors was detected.

ACKNOWLEDGMENTS

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Alteration in Membrane Lipid Order and Composition in Metabolically Hyperactive Fatty Rat Adipocytes

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We have previously shown that adipose cells from young genetically obese Zucker rats are characterized by very high metabolic activity together with an increase in a wide range of membrane-mediated functions. The aim of the present study was to examine whether the physical properties of the membranes and the composition of the membrane lipids were altered in these cells. Plasma membranes and two intracellular membrane fractions were prepared by differential ultracentrifugation from inguinal adipose cells of 30-day-old obese (fa/fa) and lean (Fa/fa) littermates. The lipid order as measured by steady-state fluorescence polarization of diphenylhexatriene used as probe was markedly decreased in the plasma membranes of obese rat adipose cells. Consistent with this, the cholesterol-to-phospholipid ratio was significantly decreased, and the degree of unsaturation of the phospholipid fatty acids was significantly increased. In intracellular membranes, none of these parameters were altered by the different genotype. In fat cells from obese rats, both plasma and intracellular membranes exhibited a 2-fold decrease in the ratios of n-6/n-3 fatty acids mainly due to an enrichment in docosahexaenoic acid (22:6n-3). The data show that the fatty genotype is a determinant of membrane lipid order and composition in adipose cells. The alterations reported here for young obese Zucker rat adipocytes might be related to the metabolic hyperactivity of these cells.

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The obesity of the (fa/fa) rat, first described by Zucker and Zucker (1), is due to a single recessive autosomal gene mutation, the nature of which remains unknown. This rat develops a syndrome which shares many features with human obesity, which recently has been shown to also have a genetic etiology (2) and for which the Zucker rat serves as an excellent model. The first phenotypic trait that develops in the mutant rat is a hypertrophy of fat cells (3). In post-weaning 30-day-old rats, these cells reach a size four times that in lean (Fa/fa) rats. Furthermore, the cells show a very high metabolic activity together with an increase in a wide range of membrane-mediated functions. Thus, obese rat fat cells show marked hyperresponsiveness to insulin in regard to the tyrosine kinase activity of the receptor (4), glucose transport (5,6) and glucose metabolism (7). In addition, these cells display a marked increase in the specific binding of several ligands, including growth hormone (8), α2 adrenoceptor agonists (9) and adenosine (10). These apparently unrelated abnormalities could be accounted for, at least in part, by a change in lipid order and/or composition of the plasma membranes as a common determinant. Indeed, increasing evidence suggests that membrane-mediated events, including transport functions, enzymes activities and ligand/receptor interactions, are influenced by the order and the composition of their lipid environment (11–13). The aim of the present study was to examine whether the physical properties and the composition of plasma membrane lipids were altered in metabolically hyperactive fatty rat adipocytes.

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Abbreviations: DBI, double bond index; DPH, 1,6-diphenyl-1,3,5-hexatriene; HDM, high density microsomes; LDM, low density microsomes; PC, phosphatidylcholine; PE, phophatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; Tris, tris(hydroxymethyl)aminoethane.

MATERIALS AND METHODS

Animals. The Zucker rats used in this study were bred from pairs originally provided by the Harriet B. Bird Laboratory (Stow, MA). Known lean heterozygous (Fa/fa) females and homozygous (fa/fa) males were mated. From this mating, 50% of the pups are expected to be obese (fa/fa) and 50% lean (Fa/fa). The rats were housed at 22–23°C and exposed to light from 7:00 a.m. to 7:00 p.m. The pups were studied at 30 d of age and were kept on the dam diet (AO4; Unité d'Alimentation Rationnelle, Epinay/Orge, France) until two days before the experiments. The fatty acid composition of this diet has been reported previously (14). Rats were killed by decapitation, between 9:00 and 10:00 a.m.

Subcellular fractionation of isolated adipocytes. Isolated adipocytes were prepared from the inguinal fat pads of approximately 35 lean and 35 obese rats by collagenase digestion. Cells were homogenized in a buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid and 255 mM sucrose, pH 7.4, at 4°C, and the homogenates were centrifuged at 16,000 × *g* max for 15 min. Plasma membrane fractions were prepared from the 16,000 × *g* pellet by centrifugation on a Percoll/sucrose gradient (15). High density microsomes (HDM) and low density microsomes (LDM) were prepared by differential ultracentrifugation of the 16,000 × *g* supernatant, as described previously (5). Proteins were determined using the Bio-Rad assay and bovine serum albumin as standard. Approximately 4-fold greater amounts of protein were recovered in membrane fractions from obese rat adipocytes as compared to those from lean littermates. Typically, the protein recovery was 1.3, 2.1 and 2.8 mg/ten obese rats compared to 0.33, 0.46 and 0.73 mg/ten lean rats for the plasma membrane, HDM and LDM, respectively. To assess the reproducibility of the cell fractionation procedure, marker enzyme activities, including 5'-nucleotidase and galactosyltransferase activities, were routinely measured, as previously described (5).

Adipocyte membrane fluidity. Adipocyte membrane lipid order was monitored by steady-state fluorescence

polarization measurements using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (16). Membranes were incubated for 45 min at 37°C in 1 mL of the homogenization buffer, in the presence of DPH at a final concentration of 10^{-7} M. Membrane proteins were adjusted to ensure a DPH/phospholipid molar ratio in the order of 1/200. Due to large variations in the phospholipid-to-protein ratios among the different membrane species (see Table 2), 20 to 100 μg of protein were used. The turbidity of the samples was always below 0.150 absorbance units at 360 nm (excitation wavelength of DPH). Steady-state fluorescence polarization measurements were made at 37°C on a SLM 8000 spectrofluorimeter (Urbana, IL). The excitation wavelength was 360 nm, and a 418 nm cutoff filter (rejecting more than 99.99% of the excitation light) was used for emission. Five measurements were averaged for each sample. The background without the probe was measured under identical conditions. The fluorescence polarization value was calculated according to $(I_{pa}-I_{pe})/(I_{pa}+I_{pe})$, where I_{pa} and I_{pe} are the fluorescence intensities emerging parallel and perpendicular to those of the original light source. This parameter gives an estimate of the resistance of DPH to rotational motion within the membrane lipid phase and is taken as a measure of the average orientational order of the lipid matrix (16–19).

Lipid composition of adipocyte membrane. Total lipids were extracted according to Folch *et al.* (20), from individual or pooled membrane preparations containing at least 1 mg of protein. Cholesterol was determined by the method of Liese *et al.* (21), and total phospholipid by the method of Bartlett (22). The membrane content in the five major phospholipid classes was determined by two-dimensional thin-layer chromatography followed by phosphorous assay of the fractions. The fatty acid composition of total phospholipid was determined by gas–liquid chromatography of the fatty acid methyl esters using a Carlo Erba (Milan, Italy) 4180 gas–liquid chromatograph, equipped with a flame-ionization detector. Authentic fatty acid methyl esters were used to assign fractions based on their retention times (14).

Statistical analysis. Results are expressed as mean ± standard error of the mean (SEM); *P* values were obtained using the unpaired Student *t*-test.

RESULTS

Fluorescence polarization of the DPH probe was determined in the plasma membrane and in two intracellular membrane fractions (HDM and LDM) from lean and obese Zucker rat adipose cells (Table 1). This parameter was markedly decreased in the plasma membranes from obese as compared to lean rat fat cells, indicating a lower lipid order. In contrast, there was no effect of genotype on DPH fluorescence polarization values in the intracellular membranes. Independent of the genotype, the intracellular membranes gave DPH fluorescence polarization values that were significantly lower than those for the plasma membranes.

The phospholipid and cholesterol contents of the subcellular membrane fractions from Zucker rat adipose cells are shown in Table 2. The phospholipid-to-protein ratio was similar in the plasma membranes from lean and obese rat adipocytes. By contrast, the cholesterol concentration was two times lower in obese than in lean rat adipocyte plasma membrane, resulting in a lower cholesterol/phospholipid ratio. In intracellular membranes, the amounts of lipid (phospholipid and cholesterol) per mg of membrane protein were markedly lower than in the plasma membranes of the same cells, but similar between the two groups.

TABLE 1

Steady-State DPH Fluorescence Polarization in Three Subcellular Membrane Fractions from 30-day-old Zucker Rat Adipocytes[a]

	Fluorescence polarization	
	Lean (Fa/fa)	Obese (fa/fa)
Plasma membranes (11)	0.215 ± 0.009	0.185 ± 0.007[b]
HDM (12)	0.127 ± 0.007[c]	0.130 ± 0.007[c]
LDM (12)	0.146 ± 0.008[c]	0.147 ± 0.008[c]

[a]Values are means ± SEM for the number of separate membrane preparations shown in parentheses. Abbreviations: DPH, 1,6-diphenyl1,3,5-hexatriene; HDM, high density microsomes; LDM, low density microsomes.

[b]Differs significantly ($P < 0.05$) from lean rat adipocyte plasma membranes.

[c]Differs significantly ($P < 0.01$) from the plasma membranes of the same cells.

The data in Table 1 and Table 2 suggest that a relationship exists between the value of DPH fluorescence polarization and the cholesterol/phospholipid ratio. By plotting individual data, we found a positive correlation ($r = 0.46$, $n = 22$, $P < 0.05$) between the two parameters, in agreement with previous reports (23,24).

The proportions of the five major phospholipid classes in adipocyte membrane fractions are shown in Table 3. The phospholipid composition of plasma and intracellular membranes was quite similar in the two groups, except for a significant decrease in the pool phosphatidylserine (PS) + phosphatidylinositol (PI) in the plasma membranes from obese rat adipocytes.

The fatty acid composition of plasma membrane phospholipids is shown in Table 4. The relative amounts of saturated and monounsaturated fatty acids were not significantly altered by the fatty genotype. By contrast, significant changes became apparent between the two groups in the polyunsaturated fatty acid levels. In obese as compared to lean rat fat cells, the proportion of n-6 fatty acids tended to be lower, whereas the amounts of n-3 fatty acids were significantly increased, with a particularly high contribution from docosahexaenoic acid (22:6n-3). Thus, the ratio of n-6/n-3 fatty acids was markedly decreased (2-fold) in the plasma membranes of obese rat adipocytes. This feature was also seen in the intracellular membranes, where the ratios of n-6/n-3 fatty acids were 4.5 and 5.0 in obese rat adipocyte compared to 10.8 and 8.2 in lean rat adipocyte HDM and LDM, respectively (means of two experiments). In addition, the degree of unsaturation of fatty acids, as measured by the double bond index (DBI), was significantly

TABLE 2

Cholesterol and Phospholipid Contents of Three Subcellular Membrane Fractions from 30-day-old Zucker Rat Adipocytes[a]

		Zucker rat adipocytes	
		Lean (mg/mg)	Obese (mg/mg)
Phospholipid/protein	Plasma membranes	1.13 ± 0.08 (4)	0.97 ± 0.07 (6)
	HDM	0.55 ± 0.05 (5)[c]	0.58 ± 0.04 (7)[c]
	LDM	0.20 ± 0.03 (5)[c,d]	0.19 ± 0.03 (7)[c,d]
Cholesterol/protein	Plasma membranes	0.46	0.24 ± 0.04 (4)
	HDM	0.13	0.11 ± 0.02 (5)[c]
	LDM	0.05	0.04 ± 0.01 (5)[c,d]
Cholesterol/phospholipid	Plasma membranes	0.38 (2)	0.25 ± 0.01 (5)[b]
	HDM	0.24 (2)	0.18 ± 0.03 (6)
	LDM	0.25 (2)	0.22 ± 0.01 (6)

[a]Values are means ± SEM for the number of separate membrane preparations shown in parentheses. LDM, low density microsomes. In lean rat adipose cells, cholesterol was measured on a pooled sample from five separate membrane preparations.

[b]Differs significantly ($P < 0.05$) from lean rat adipocyte plasma membranes.

[c]Differs significantly ($P < 0.05$) from the plasma membranes of the same cells.

[d]Differs significantly ($P < 0.05$) from high density microsomes (HDM) of the same cells.

TABLE 3

Phospholipid Composition of Three Subcellular Membrane Fractions from 30-day-old Zucker Rat Adipocytes[a]

	Plasma membranes		HDM		LDM	
	Lean (3)	Obese (6)	Lean (2)	Obese (6)	Lean (2)	Obese (6)
Phospholipid	(% of total in weight)					
PC	37.7 ± 2.1	44.7 ± 0.9	53.5	54.1 ± 3.3	45.1	51.3 ± 4.0
PE	28.1 ± 3.6	27.7 ± 1.0	26.8	23.6 ± 1.2	29.7	21.4 ± 2.1
PS + PI	21.7 ± 0.3	14.1 ± 1.5[b]	13.8	14.2 ± 1.7	17.3	16.1 ± 1.6
SPH	12.7 ± 2.2	13.6 ± 0.7	6.1	8.1 ± 0.7	8.1	11.2 ± 2.0

[a]Values are means ± SEM for the number of separate membrane preparations shown in parentheses. For abbreviations: See Table 2 and PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin.

[b]Differs significantly ($P < 0.05$) from lean rat adipocyte plasma membranes.

higher in the plasma membranes from obese as compared to lean rat adipocytes, but was unaffected by the genotype in intracellular membranes.

DISCUSSION

This study shows, for the first time, the effect of the fatty genotype on the physical properties and lipid composition of adipocyte subcellular membranes. The data illustrate that there is a substantial decrease in the plasma membrane lipid order in obese rats, together with a decrease in the cholesterol-to-phospholipid ratio and an increase in fatty acyl unsaturation. In contrast to plasma membranes, no change could be detected in intracellular membranes. This suggests that the regulation of adipocyte membrane lipids by the fatty genotype affects mainly, if not exclusively, the plasma membrane.

The genetic dependence of adipocyte plasma membrane lipid order and/or lipid composition has been previously shown in two strains of obese mice, ob/ob and db/db (25–27). In ob/ob mice, plasma membrane lipid order was also found to be decreased as compared to lean mice. However, in these mice there was no change in the phospholipid/cholesterol ratio or fatty acyl unsaturation (25,26). In db/db mice, the fatty acids of adipocyte plasma membranes were shown to be less unsaturated than in lean mice (27). Thus, from the studies in obese mice and based on our present data, it is clear that the lipid composition of the adipocyte plasma membrane is regulated in a genotype-specific manner.

The large changes in lipid order and composition we found in the plasma membranes of obese rat adipocytes could be implicated in the altered membrane-associated functions displayed by these metabolically hyperactive cells (4–10). The relationship between lipid order and/or composition and functional properties of membranes has been suggested by several models. Relevant observations have been made in 3T3-F442A adipocytes where a decrease in plasma membrane lipid order and in cholesterol/phospholipid ratio and an increase in phospholipid fatty acid unsaturation was observed during adipose conversion when cells undergo changes in several

TABLE 4

Fatty Acid Composition of Total Phospholipids of Adipocyte Plasma Membranes from 30-day-old Zucker Rats[a]

Fatty acid	(% of total in weight)	
	Lean	Obese
Saturated		
14:0	2.7 ± 0.3	2.7 ± 0.3
16:0	22.7 ± 3.7	26.0 ± 1.5
18:0	13.0 ± 1.7	16.4 ± 0.8
Total	42.8 ± 5.5	49.3 ± 2.1
Monounsaturated		
16:1n-7	2.3 ± 0.5	3.1 ± 0.3
18:1n-9	17.8 ± 0.3	14.6 ± 0.7
18:1n-7	2.3 ± 0.1	1.9 ± 0.1
Total	24.7 ± 0.8	21.4 ± 1.2
n-6 Polyunsaturated		
18:2n-6	14.7 ± 1.4	11.5 ± 0.8
20:3n-6	0.7 ± 0.2	0.8 ± 0.2
20:4n-6	11.5 ± 3.4	9.7 ± 0.9
22:5n-6	1.2 ± 0.4	1.1 ± 0.4
Total	29.0 ± 4.5	24.0 ± 1.7
n-3 Polyunsaturated		
22:5n-3	0.7 ± 0.1	0.7 ± 0.1
22:6n-3	1.9 ± 0.3	3.8 ± 0.3[b]
Total	3.5 ± 0.5	5.3 ± 0.4[b]
n-6 + n-3	32.4 ± 5.0	29.3 ± 1.9
n-6/n-3 ratio	8.3 ± 0.2	4.6 ± 0.3[b]
DBI	1.0 ± 0.02	1.4 ± 0.06[b]

[a]Values are means ± SEM for three and five separate membrane preparations in lean and obese rats, respectively. DBI, double bond index.

[b]$P < 0.05$ vs. adipocyte plasma membranes from lean rat.

integral membrane functions (28). In reconstituted plasma membrane vesicles, both the binding and the kinase activity of the insulin receptor (29) as well as the glucose transport activity have been shown to correlate with the degree of fatty acid unsaturation of the surrounding phospholipids (30). The Zucker rat provides a physiological model supporting the conclusion from these *in vitro* studies that decreased lipid order or increased fatty acid unsaturation is associated with increased membrane functional activity. One could speculate that changes in the physiochemical properties of the fat cell plasma membrane could affect vesicle fusion and budding involved in exocytosis, endocytosis and recycling, which, in turn, affects the quantity and exposure of receptors and transport proteins at the cell surface. It is also possible that the increase in docosahexaenoic acid, that we found in obese rat adipocytes, plays a role in the metabolic hyperactivity of these cells. Indeed, in ongoing experiments we have observed that dietary fish oil rich in n-3 fatty acids induces a marked increase in both the insulin responsiveness and the lipid storage capacity of the adipose cells when compared to the effect of dietary saturated or n-6 fatty acid (Hainault, I., Guichard, C., and Lavau, M., unpublished data).

The mechanism through which the fatty genotype influences the fatty acyl composition of the adipocyte plasma membrane is far from being clear. The fatty acid composition of phospholipids from various tissues in Zucker rats has been recently reported (14,31). A decrease in the ratios of n-6/n-3 fatty acids was observed in liver and kidney but an increase was observed in heart, and no change was seen in brain or retina. Taken together, the data thus point toward a tissue specificity of the fatty genotype effect.

In conclusion, our work demonstrates that the genetic background influences the lipid order and composition of the adipocyte plasma membrane, thereby having the potential to modify membrane-mediated control of cellular activity. The alterations revealed by the present study in adipocytes from young obese Zucker rats could be involved in the metabolic hyperactivity of these cells.

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Synthesis and Structural Analysis of Stereospecific 3,4,5-Trisubstituted γ-Butyrolactone Phospholipids

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The first synthesis of stereospecific 3,4,5-trisubstituted γ-butyrolactone phospholipids has been achieved by a ten-step sequence starting from *N,N*-dimethylacetamide (9). Alkylation of 9 with 1-bromohexane gave *N,N*-dimethyloctanamide (11), which was subjected to aldol condensation with acrolein and afforded the *erythro*-*N,N*-dimethyl-3-hydroxy-2-hexyl-4-pentenamide (6) and its *threo* isomer 7, both of which were subsequently converted to the corresponding 3-oxy(hexanoyl) esters 15 and 16. Stereospecific cyclization of diastereomeric allylic amides (6,7,15 and 16) *via* iodolactonization gave 3,4,5-trisubstituted γ-butyrolactones (19–25) in satisfactory yields (68–89%) and with high stereoselectivity (at least 6:1), except for the *threo* amide 16 (2:1). Conversion of the 6-iodo-group of the major iodolactones 23 and 24 to the corresponding 6-hydroxy lactones 31 and 32 through a wet Prévost reaction also resulted in 4-hydroxy lactones 30 and 33, and a mechanism for this reaction is proposed. Further phosphorylation of 4- or 6-hydroxy lactones 30, 31 and 32 with diphenyl chlorophosphate gave the corresponding diphenyl phosphoric acid lactone esters 34, 35 and 36, whereas 33 underwent an elimination reaction under formation of a C(3)–C(4) double bond. Hydrogenolysis of the diphenyl groups of esters 34, 35 and 36 over Adams' catalyst [platinum(IV) oxide] yielded the corresponding phosphoric acid lactone esters 38, 39 and 40, which were finally treated with choline tetraphenylborate to afford the desired lactone phospholipids 41, 42 and 43 in overall yields of 9, 30 and 18%, respectively. The structures of all new compounds were unambiguously assigned by analysis of their ^{1}H and ^{13}C one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectra obtained on a 300 MHz NMR spectrometer, and the structures were confirmed by low or high resolution mass spectrometry as well as elemental analysis. The stereoisomeric purity of the final products (98.2, 99.0 and 98.5% for 41, 42 and 43, respectively) was determined by high-performance liquid chromatography.

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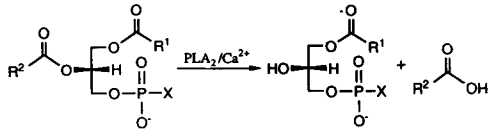
The mechanism and the inhibition of phospholipase A_2 (PLA_2) (EC 3.1.1.4) action are topics of much current biological and medical interest. PLA_2 specifically cleaves the *sn*-2 acyl bond of phospholipids and produces equimolar amounts of lysophospholipid and free fatty acid (Scheme 1) (1–4). Thus, PLA_2 also plays an important role in the release of arachidonic acid, the sole precursor for the biosynthesis of eicosanoids, that include prostaglandins, leukotrienes and thromboxanes (5–7). Eicosanoids have been implicated in the pathophysiology of many diseases, and especially those involving inflammation and allergy (6–8). Therefore, inhibition of PLA_2 is a logical approach to the treatment of these conditions.



$R^1, R^2 = C_mH_{2m+n}$, m = 13, 15, 17, 19; n = 1, -1, -3, -5, -7, etc...
X = -OH, $-OCH_2CH_2N^+(CH_3)_3$, $-OCH_2CH_2NH_2$, etc...

SCHEME 1

In the design and synthesis of potential PLA_2 inhibitors, phospholipid analogues have attracted considerable attention. It is known that for a compound to serve as a PLA_2 substrate, a fatty acid ester must be bonded vicinally to a phosphate ester (9). Noticeable progress has been made in the preparation of acyclic phospholipids as PLA_2 inhibitors by systematically modifying the structure of phosphatidylcholine (9), using computer assisted molecular modeling (10–12) and total synthesis of various phospholipid analogues (13–20). However, few studies have been undertaken with specifically designed and conformationally restricted phospholipids. By this approach, one restricts the flexibility of a substrate phospholipid and permits PLA_2 to select the preferred orientation of the side chain(s), so that the orientation preferred by PLA_2 can be determined.

One decade ago, Hancock *et al.* (21) pioneered the synthesis of cyclopentanoid analogues of phosphatidylcholine and investigated the physical properties of these compounds. Following this work, Barlow *et al.* (22) prepared a short acyl-chain phosphatidylcholine analogue, the glycerol backbone of which was replaced by the all *trans* cyclopentane-1,2,3-triol; this compound was tested as a substrate for PLA_2 from *Naja naja naja* venom. Moreover, Magolda and Galbraith (23) reported a series of new 1′-(3′,5′-disubstituted)pyrrolidinyl-alkyl/arylcarboxamide phospholipids (so called proximal isomer and distal isomer), which were tested as inhibitors of porcine pancreatic PLA_2. Campbell and co-workers (12), starting from 4-hydroxymethyl-γ-butyrolactone, synthesized two conformationally restricted 3,4-disubstituted γ-butyrolactone phospholipid analogues, namely the 3-arachidonyl substituted lactone, which exhibited an IC_{50} (inhibitory concentration to produce 50% inhibition) value of 64 μM against PLA_2 in a cell-free preparation and 44 μM in a macrophage test, and the 3-butanoyl substituted lactone that showed no inhibitory activity toward the enzyme. However, 3,4,5-trisubstituted

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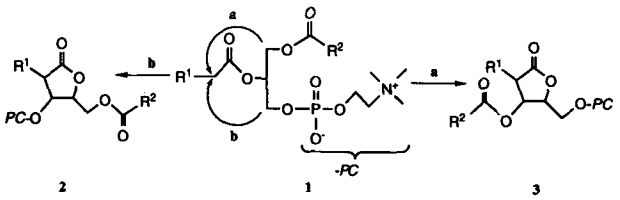
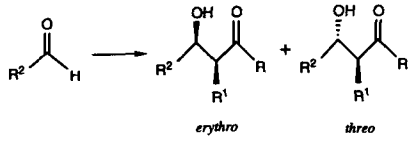
Abbreviations: ADEPT, automatic distortionless enhancement by polarization transfer; DMAP, 4-dimethylaminopyridine; HETCOR, heteronuclear correlation; HPLC, high-performance liquid chromatography; HRMS, high resolution mass spectrometry; IR, infrared; *J*, coupling constant(s); LDA, lithium diisopropylamide; LRMS, low resolution mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; PLA_2, phospholipase A_2; THF, tetrahydrofuran; TLC, thin-layer chromatography.



SCHEME 2



SCHEME 4

γ-butyrolactone phospholipids, in which the glycerol backbone is part of the γ-butyrolactone ring and which represent novel types of conformationally restricted substrate analogues, have not been studied so far. Cyclization of C(1) or C(3) of the acyclic glycerophospholipid **1** to the α-carbon of the C(2) acyl side chain, as indicated by arrows **a** and **b** (Scheme 2), allows for two regioisomeric possibilities. Based on regioisomers **2** and **3**, a series of stereoisomers can be designed, and this new class of 3,4,5-trisubstituted γ-butyrolactone phospholipids (**41, 42** and **43**) has been synthesized. The inhibitory activity of the compounds is presently under investigation, and the results will be published in a separate paper. In the present paper, we wish to report on the synthesis, the structural assignment, and on the stereochemistry of these novel stereospecific phospholipid analogues.

RESULTS AND DISCUSSION

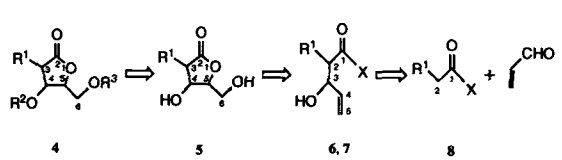
Retrosynthesis. The preparation of the target phospholipid lactones **4** (R_1 = alkyl, R_2/R_3 = ester/choline phosphate or choline phosphate/ester, respectively) is based on the regioselective manipulation of dihydroxylactone **5** (Scheme 3). Access to intermediate **5** is expected to arise *via* cyclization (such as iodolactonization) of *erythro* diastereomer **6** or *threo* isomer **7** [X = $N(CH_3)_2$ or OCH_3; R_1 = alkyl]. The intermediates (**6** and **7**) could be derived from **8** *via* aldol condensation of the appropriate carboxylate derivative with acrolein (24,25), a process that has been studied widely over the past two decades.

Synthesis. In practice, the diastereomeric allylic amides **6** and **7** were prepared in four steps (67% overall yield), starting from *N,N*-dimethylacetamide (**9**). Thus **9** was converted to its enolate with lithium diisopropylamide (LDA), which after treatment with 1-bromohexane afforded *N,N*-dimethyloctanamide (**11**). The enolate of **11** was condensed with acrolein to form diastereomers **6** and **7** (*erythro/threo*, 2:1) in 66% yield after chromatographic purification (24,26).

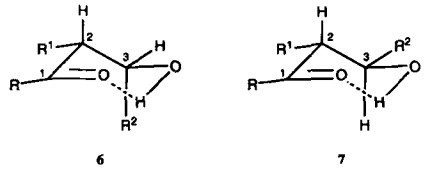
There is some divergence in the literature with regard to the *erythro* and *threo* designation for vicinally asymmetric centers in acyclic molecules. In the present paper, we use the convention of Heathcock *et al.* (24,25). When the backbone of an aldol is written in an extended (zigzag) fashion, and when the α-alkyl substituent and β-hydroxy substituent both extend toward the viewer or away from the viewer, this is the *erythro* diastereomer. When the α-alkyl group extends toward the viewer, but the β-hydroxy group is directed away or *vice versa*, as illustrated in Scheme 4, this is the *threo* diastereomer. In order to make stereochemical assignments of the allylic hydroxy amides **6** and **7**, the ^1H nuclear magnetic resonance (NMR) spectra of two isomers were examined. The NMR coupling constant (J), $J_{H2,H3}$ is smaller for isomer **6** (J = 4.2 Hz) than for isomer **7** (J = 7.5 Hz). This could be explained (Scheme 5) by postulating that isomer **7** has pseudo *trans* diaxial protons H-2 and H-3 on the hydrogen-bonded, six-membered ring in chair conformation with the maximum number of substituents in equatorial positions (26). The R_f value in thin-layer chromatography (TLC) of isomer **6** (0.26) was higher than that of isomer **7** (0.11; hexane/ethyl acetate, 3:1, vol/vol), suggesting that isomer **6** may be hydrogen bonded to a greater extent and may be less capable of interacting with the stationary phase than the more polar isomer **7** (27). This can be confirmed by evaluating the NMR coupling constants (26). Based on these considerations, the β-hydroxy amide isomer **6** was assigned the *erythro* configuration, and **7** was determined to be the *threo* isomer.

Esterification of the allylic hydroxy groups of **6** and **7** by treatment with hexanoic anhydride and catalytic 4-dimethylaminopyridine (DMAP) at room temperature gave the corresponding 3-oxy(hexanoyl) esters **15** and **16** in over 95% yield. In order to study the stereochemistry of 5-iodomethyl-γ-butyrolactones in the next step, the synthesis of amide derivatives **12, 13, 14, 17** and **18** was undertaken in a similar manner beginning with *N,N*-dimethylpropionamide **10** (Scheme 6).

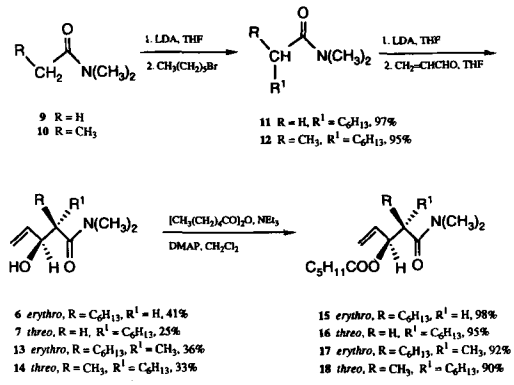
Stereospecific cyclization of diastereomers **6** and **7** to the γ-butyrolactones was accomplished through iodolactonization, which has been extensively utilized (28–30) for the efficient and highly stereocontrolled preparation of substituted γ-butyrolactones (31–36). For the trisub-



SCHEME 3



SCHEME 5



SCHEME 6

stituted lactones, the α and β terminology of IUPAC was used to express the stereochemical relationship of the substituents on the lactone ring. Tamaru *et al.* (29) reported that in structurally simpler systems, α-alkyl-β-oxy-substituted pentenamides cyclized with a high degree of stereocontrol and/or greater yield when compared to the analogous carboxylates (30). As part of our efforts in this area, we investigated the iodolactonization of diastereomeric allylic 3-hydroxy amides **6, 7, 13, 14** and their hexanoyl esters **15, 16, 17** and **18**. In order to more fully elucidate the stereostructure-activity relationship in this reaction, novel 3,4,5-trisubstituted γ-butyrolactones **19, 20, 21, 22, 23, 24, 25** and **26, 27, 28, 29, 44, 45** were prepared by treatment of the respective amides with iodine in aqueous tetrahydrofuran (THF) at ambient temperature. Theoretically, the generation of a new chiral center (such as at C-5 in a 5-iodomethyl-γ-butryolactone) could lead to two possible diastereomers. In practice, *erythro* **6** underwent highly stereoselective cyclization, giving **19** and **20** as a 7:1 mixture of C-5 epimers (68% yield). By a similar process, iodolactones **21/22** (6:1) were produced from the *threo* isomer **7** in 74% yield. Iodolactonization of **15** and **16** was accomplished under the same conditions. *Erythro* isomer **15** gave almost exclusively **23** [(3α,4β,5β)/(3α,4β,5α), >95:5] in 86% isolated yield. *Threo* compound **16** gave a mixture [**24/25**, (3α,4α,5β)/(3α,4α,5α), 2:1] with low stereoselectivity but very high yield (89%). Two other pairs of diastereomers (**13/14** and **17/18**) were prepared following the method described for the synthesis of **6** and **7**. By iodolactonization of amides **13, 14, 17** and **18**, iodolactones **26, 27, 28** and **29** were obtained with high stereoselectivity in good to excellent yields (70–98%). The results of these iodolactonization experiments are summarized in Table 1.

TABLE 1

Iodolactonization[a] of Erythro/Threo Allylic Amides

Starting amide	Iodolactones		A/B[b]	Yield
6	**19** (3α,4β,5β), R^1=+$(C_2)_5CH_3$, R^2=+H, R^3=H, R^4=OH	**20** (3α,4β,5α), R^1=+$(CH_2)_5CH_3$, R^2=H, R^3=H, R^4=OH	7:1 ii	68%
7	**21** (3α,4α,5β), R^1=$(CH_2)_5CH_3$, R^2=H, R^3=OH, R^4=H	**22** (3α,4α,5α), R^1=$(CH_2)_5CH_3$, R^2=H, R^3=OH, R^4=H	6:1 ii	74%
13	**26** (3α,4α,5α), R^1=CH_3, R^2=$(CH_2)_5CH_3$, R^3=H, R^4=OH	None isolated	>95:5 ii	98%[c]
14	**27** (3α,4α,5α), R^1=CH_3, R^2=$(CH_2)_5CH_3$, R^3=H, R^4=OH	**45** (3α,4α,5β), R^1=CH_3, R^2=$(CH_2)_5CH_3$, R^3=H, R^4=OH	10:1 i	95%
15	**23** (3α,4β,5β), R^1=$(CH_2)_5CH_3$, R^2=H, R^3=H, R^4=$OCO(CH_2)_4CH_3$	None isolated	>95:5 i,ii	86%
16	**24** (3α,4α,5β), R^1=$(CH_2)_5CH_3$, R^2=H, R^3=$OCO(CH_2)_4CH_3$, R^4=H,	**25** (3α,4α,5α), R^1=$(CH_2)_5CH_3$, R^2=H, R^3=$OCO(CH_2)_4CH_3$, R^4=H	2:1 i	89%
17	**28** (3α,4β,5β), R^1=$(CH_2)_5CH_3$, R^2=CH_3, R^3=H, R^4=$OCO(CH_2)_4CH_3$	None isolated	>95:5 i	81%
18	**29** (3α,4α,5α), R^1=CH_3, R^2=$(CH_2)_5CH_3$, R^3=H, R^4=$OCO(CH_2)_4CH_3$	**44** (3α,4α,5β), R^1=CH_3, R^2=$(CH_2)_5CH_3$, R^3=H, R^4=$OCO(CH_2)_4CH_3$	4:1 i	86%

[a]Iodolactonization condition: I_2, (1.5 eq.), H_2O (20.0 eq.), tetrahydrofuran, rt, 12–24 h.

[b]As determined by nuclear magnetic resonance (i) or isolated yield (ii).

[c]Corrected for recovered olefin (44%) after 16 h.

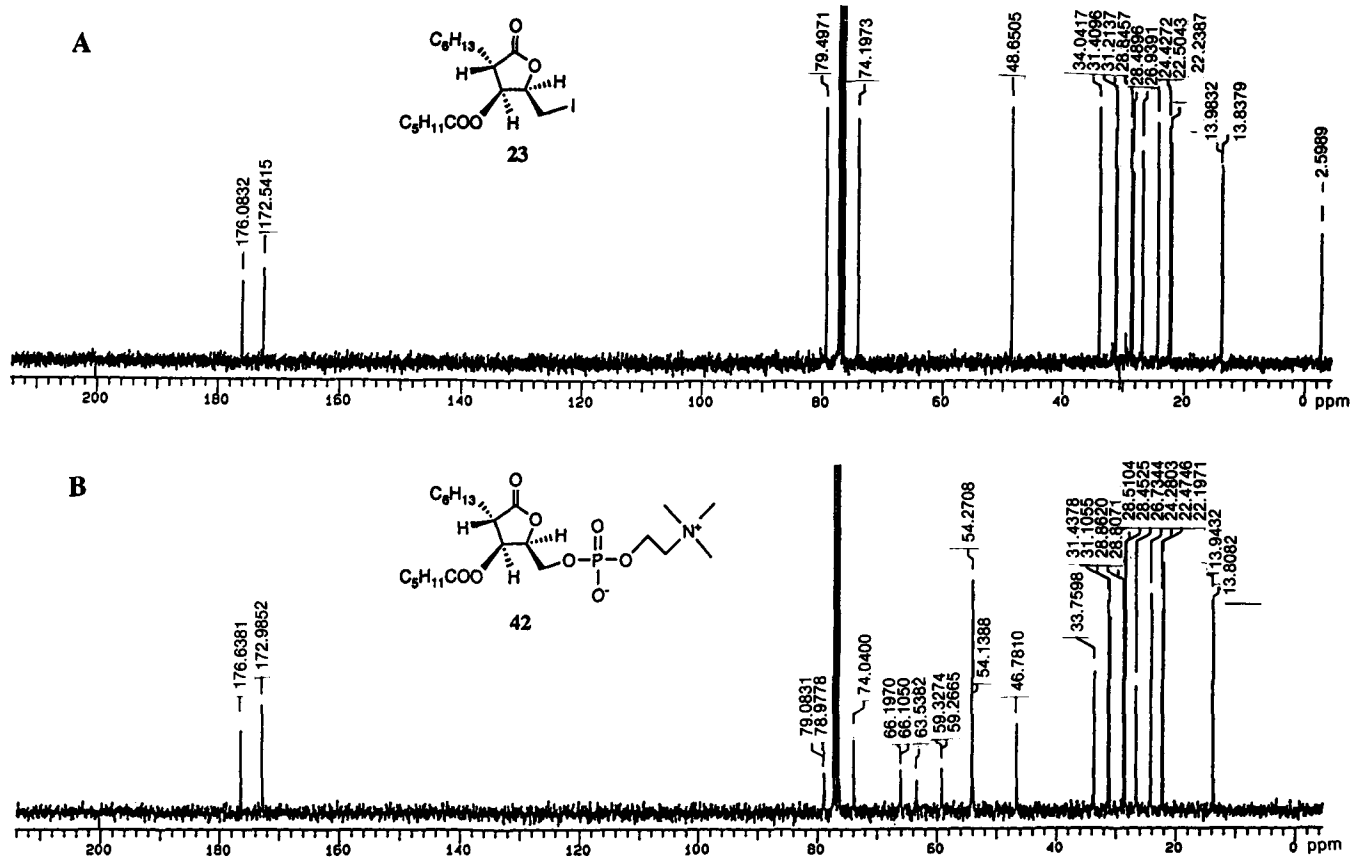


FIG. 1. ^{13}C Nuclear magnetic resonance spectra (75.47 MHz, chloroform-*d* for A, chloroform-*d*/methanol-d_4 (3:1, vol/vol) for B of A iodolactone 23 and B lactone phospholipid 42.

In order to arrive at unambiguous structural assignments for all iodolactonization products, the spectral characteristics of each of the iodolactones were analyzed in detail. The characterization of iodolactone **23**, one of the key intermediates, is chosen here to illustrate this. Two intense absorptions were seen in the infrared (IR) spectra of **23** at 1785 and 1748 cm^{-1}, suggesting the presences of carbonyls, of a saturated γ-lactone and an aliphatic ester, respectively. The electron impact mass spectrum of **23** showed an [M^+ – 127] ion peak at *m/e* 297, which indicated a molecular weight of 424, corresponding to **23** losing iodide. McLafferty rearrangements of either the 4-oxy(hexanoyl) ester or the 3-hexyl side chain resulted in the fragmentation ions at *m/e* 241 [M^+ – I-$C_2H_5CH{=}CH_2$] or *m/e* 213 [M^+ – I-$C_4H_9CH{=}CH_2$], respectively, while α-cleavage of the hexanoyl from the 4-oxygen gave rise to the fragmentation ion peak *m/e* 198 [M^+ – I-$C_5H_{11}CO^+$]. The ^{13}C NMR spectrum (Fig. 1A) of **23** shows the characteristic signals of 3-hexyl-4-oxy(hexanoyl)-5-iodomethyl-γ-butyrolactone at δ 176.1 (C-2), 172.5 (C-1′), 79.5 (C-5), 74.2 (C-4), 48.7 (C-3) and –2.6 (C-6), plus two terminal methyl carbons (C-12 and C-6′) at δ 13.8 and 14.0 ppm, respectively, and 9 methylene carbons in the region of 22.2–34.0 ppm. The representative protonated carbons (C-5, C-4, C-3 and C-6) in the two-dimensional heteronuclear correlation (HETCOR) spectrum (Fig. 2A) were correlated with the proton signals in the ^1H NMR spectrum of **23** at δ 2.63, 3.32/3.38, 4.76 and 5.28 ppm for H-3, H-6A/H-6B, H-5 and H-4, respectively. Furthermore, the structure of **23** was in good agreement with the results of high-resolution mass spectrometry (HRMS). The structural assignment of the other iodolactones was accomplished similarly.

The stereochemistry of the iodolactones was determined by 300-MHz nuclear Overhauser effect spectroscopy (NOESY) and based on vicinal coupling constants of the γ-butyrolactone ring protons. It was observed that when both vicinal hydrogens were in either α or β stereorelationship, the *J* value was at least 3 Hz or larger. If one of the adjacent protons was α and the other was β, or *vice versa*, the *J* value was smaller, ranging from *ca.* 1–2 Hz (28). Strong NOESY interactions were evident between hydrogen atoms that were both in an α or β relationship, and in case of geminally substituted lactones derived from **13, 14** and **18**, where both β-H-4 and β-H-5 clearly showed interaction with the 3β-methyl group, as in iodolactones **26, 27, 29** (Fig. 3A) (28). However, when the 3-methyl group was substituted in α phase while H-4 and H-5 were still in β phase, such as in the lactone **28**, H-4 interacted with two protons (H-7A and H-7B) on the 7-methylene carbon (which was adjacent to C-3), whereas H-5 interacted with only one, as shown in Figure 3B. Furthermore, the H-4 and H-5 protons of iodolactone **23** showed the same interaction pattern (Fig. 3C) as observed for **28**; thus, lactone **23**

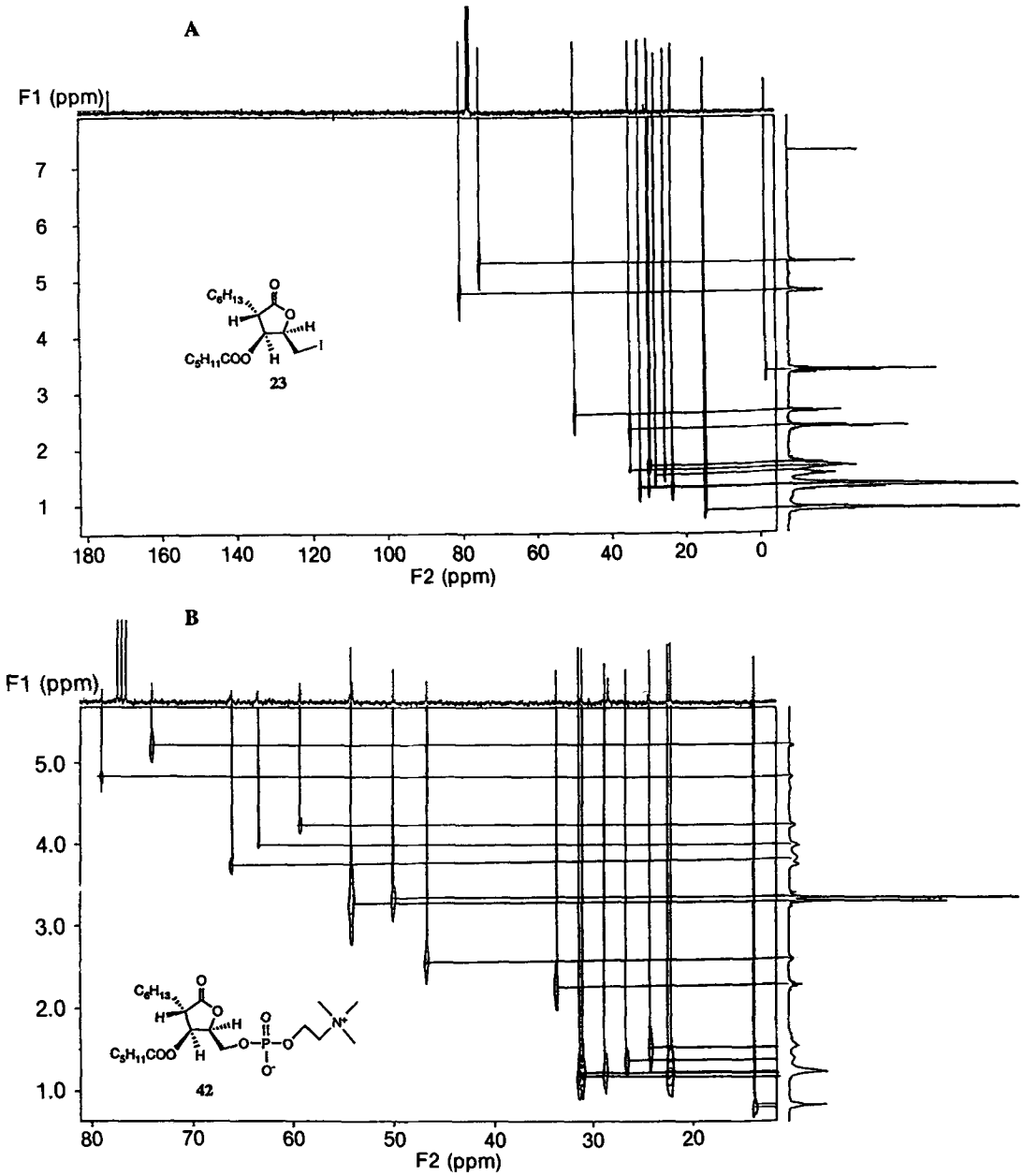


FIG. 2. Nuclear Overhauser effect spectra (300.13 MHz, chloroform-*d*) of the iodolactones A, 29, B, 28 and C, 23.

was assigned to have the (3α,4β,5β)-trisubstituent stereorelationship, which was consistent with the coupling constants $J_{H\text{-}3,H\text{-}4}$(1.3 Hz) and $J_{H\text{-}4,H\text{-}5}$(4.5 Hz) in the ^1H NMR spectrum of **23**. The stereochemical relationship of 3,4,5-trisubstituents of other iodolactones was elucidated in a similar manner. As a further confirmation of the lactone stereochemistry, molecules having a free hydroxy group were converted to their corresponding hexanoyl esters (37) and compared to previously characterized authentic samples prepared independently by iodolactonization. In general, the total yields of the iodolactonization products were good to excellent, and with only one exception (**16**) each isomer stereospecifically cyclized to a preparatively useful extent (at least 4:1). It is significant that, in most cases, both isomers of a given diastereomeric amide cyclized with a higher level of stereocontrol than had been observed in other studies with related compounds (29,30). The general tendency (29) for the newly created stereocenter at C-5 to be *cis* to the oxygen substituent at C-4 was apparent in each instance except for **16**, and the basis for this reversal is unknown at the present time. The reason for the increased stereoselectivity observed during cyclization of the *threo* derivatives of the hexanoylated amides is likewise obscure. It does not appear to be a function of either the amount of water present or of the solvent employed. Analogous results were obtained when diethyl ether was replaced by THF, or when the amount of water in THF was increased to 40 equivalents or decreased to 5 equivalents. It is possible that there were

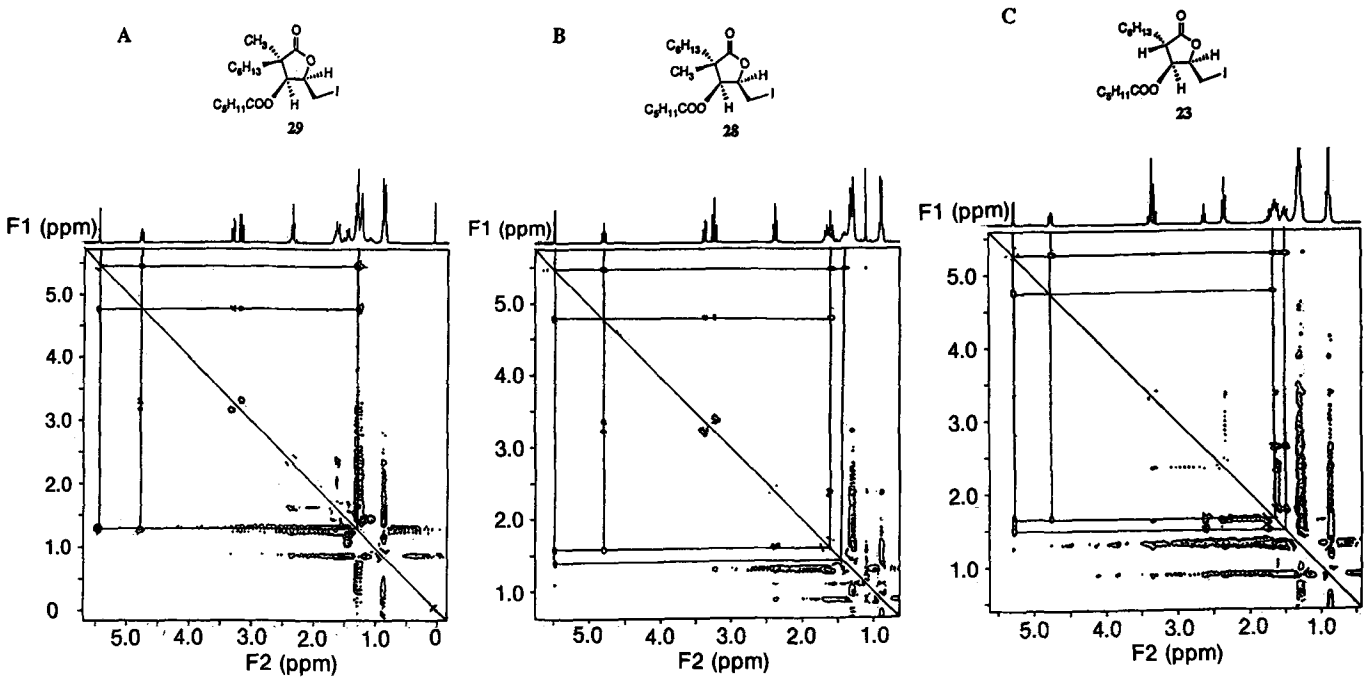
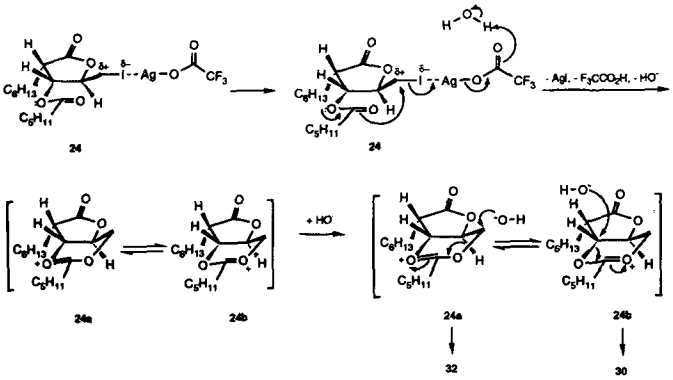


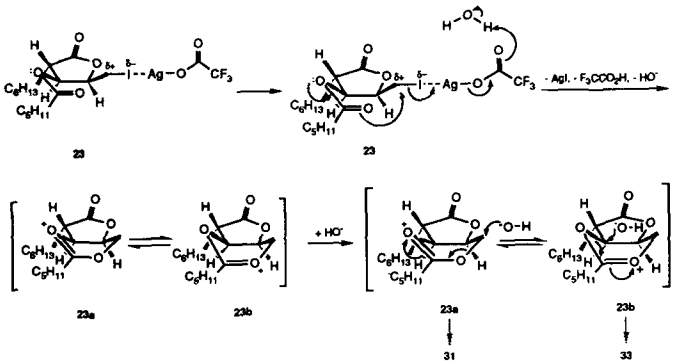
FIG. 3. Heteronuclear correlation spectra (300.13 MHz for ^1H and 75.47 MHz for ^{13}C, chloroform-*d* for A, chloroform-*d*/methanol-d_4 (3:1, vol/vol) for B of A iodolactone 23 and B lactone phospholipid 42.

subtle changes induced by the lipophilic substituents in C(2) and/or C(3) positions of the allylic amide that sufficiently influenced the conformational bias (and hence lowered E_{act}) in favor of one of the rotamers, leading to the observed results.

The final step in the synthesis was the introduction of the choline phosphate side chain at the 4- or 6-hydroxy group of the lactones. Thus, the 6-iodo substituent of the major products **23** and **24** that were obtained by iodolactonization was converted to the corresponding 6-hydroxy function *via* a wet Prévost reaction (38,39) by stirring with silver(I)trifluoroacetate and water in nitromethane (33). Mixtures of two alcohol products **30/32** and **31/33** were obtained, respectively. Mass spectral analysis suggested that the products were regioisomeric in both cases. IR, ^1H NMR and ^{13}C NMR spectra of compounds **30, 31, 32** and **33** indicated that all four products contained both 4- and 6-oxygenated species. However, the ^1H NMR spectra were of little assistance in the assignment, because the key resonance signals were not resolved at 300 MHz in $CDCl_3$ solution. In order to shed more light on the reaction, the chromatographic behavior of the compounds on TLC was investigated further. When compounds **30, 31, 32** and **33** were run in the same solvent system (hexane/ethyl acetate, 3:1, vol/vol), compounds **30** (R_f = 0.30) and **33** (R_f = 0.32) showed higher R_f values than did their isomers **31** (R_f = 0.22) and **32** (R_f = 0.18). Due to differences in steric hindrance, the R_f values of the 6-hydroxy lactones would be expected to be lower than those of the 4-hydroxy compounds, because the primary alcohols would interact more strongly with the adsorbent than a secondary alcohol would. Thus, the less polar hydroxy lactones **30** and **33** were assigned the 4-hydroxy-6-oxy(hexanoyl) ester lactone structure, while the more polar regioisomers **31** and **32** were designated as 6-hydroxy-4-oxy(hexanoyl) derivatives. All products obtained in this reaction from **23** and **24** were useful intermediates for the conversion to the desired target lactone phospho-



SCHEME 7



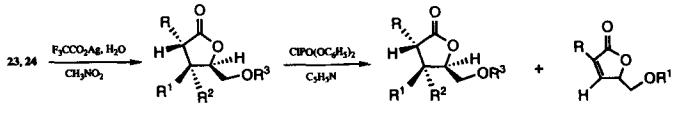
SCHEME 8

lipids. The mechanism we propose for the wet Prévost reaction is shown in Schemes 7 and 8. When the silver atom of silver trifluoroacetate approaches the 6-iodide, it polarizes the bond between C-6 and iodine, resulting in the formation of a silver iodine complex. The migration of the electron pair at the 4-oxygen then initiates a nucleophilic attack, in which the carbonyl oxygen of both α- and β-4-oxy(hexanoyl) attacks the polarized electron-deficient carbon (C-6) from either the α phase (in the case of **24**) or the β phase (in the case of **23**) in the lactone planes, followed by a series of electron transfers that lead to the chair-like (**24a** and **24b**) or boat-like (**23a** and **23b**) six-membered ring (4,5) conjugated γ-butyrolactone intermediates, together with other products, including insoluble silver iodide, which moves the reaction completely toward the product side. The hydroxy ion (HO^-), which is generated nucleophilicly, attacks C-4 from the β phase (in the case of **24b**) or the α phase (in the case of **23b**) of both the lactones and six-membered rings and then opens the chair-like or boat-like six-membered rings by an S_{N2} reaction mechanism leading to 4-hydroxy-6-oxy(hexanoyl) ester lactones **30** and **33** with inversion (Walden inversion) of the configuration at C-4. On the other hand, 6-hydroxy-4-oxy(hexanoyl) ester lactones **31** and **32** (from the boat-like intermediate **23a** and the chair-like intermediate **24a**, respectively) are produced when the hydroxy ion nucleophilicly attacks the C-6 positions, while in both cases the stereochemistry at C-4 remains the same as in the 5-iodomethyl-γ-butyrolactones used as starting materials. The conversion was effected in high yields (98% from **23**, and 86% from **24**) but, not unexpectedly, each iodolactone gave two hydroxy lactone products, namely **31/33** (7:3) and **32/30** (5:3) with the stereochemistry at C-4 either identical to that of the starting material (**31** and **32**) or with inverted configuration (**30** and **33**) (Scheme 9).

In order to phosphorylate the 4- or 6-hydroxyl group of these lactones, the usefulness of the three-step method described by Magolda and Johnson (40) was investigated. It involves the treatment of the hydroxy compound with phosphorus oxychloride in triethylamine and anhydrous diethyl ether at 0°C to give a dichlorophosphate ester followed by exposure of the dichlorophosphate to ethylene glycol in the presence of triethylamine to effect the conversion to the corresponding cyclic phosphate. Final heating in acetonitrile with trimethylamine in a sealed tube for 30 h at 75°C affords the phosphocholine. However, our starting lactones decomposed under these conditions. An alternate phosphorylation approach that utilizes a two-step protocol, as outlined by Yuan *et al.* (18), proved more successful. The hydroxy lactones (**30, 31** and **32**) were treated with diphenyl chlorophosphate in anhydrous pyridine and afforded the corresponding diphenyl phosphoric lactone esters in good to excellent yields (70, 82 and 95% for compounds **35, 36** and **34**, respectively), whereas compound **33** gave exclusively a C(3)-C(4) elimination product (**37**, 96% yield) (Scheme 9). Several unsuccessful attempts were made to obtain the 4-oxy(phosphate) of **33**, that involved the use of different solvents, such as anhydrous *N,N*-dimethylformamide, methylene chloride and THF, as well as lower reaction temperatures and the use of different phosphorylating reagents. One possible reason for this undesired reaction taking place may be the *trans*-relationship between the 3-proton and 4-hydroxy group, which may facilitate elimination of the sterically hindered oxygen substituent.

Removal of the diphenyl moieties of diphenyl phosphoric acid lactone esters was accomplished in high yield (>90%) by hydrogenation of **34, 35** and **36** in the presence of Adams' catalyst [platinum(IV)oxide, PtO_2] with methanol as solvent at room temperature for 12–36 h (Scheme 10). The last step was the introduction of the choline side chain. Compounds **38, 39** and **40** were treated with choline tetraphenylborate and 2,4,6-triisopropylbenzenesulfonyl chloride in anhydrous pyridine at 35°C for 4 h (18). This afforded the target 3,4,5-trisubstituted lactone phospholipids **41, 42** and **43** in 36, 68 and 43% yields, respectively, after chromatographic purification (Scheme 10).

The ^{13}C NMR spectrum (Fig. 1B) of the lactone phospholipid **42** derived from iodolactone **23** showed signals at δ 176.6 (C-2), 172.9 (C-1′), 79.0 (C-5), 74.0 (C-4), 63.5 (C-6), and at 46.8 (C-3), at 66.2, 59.3 and 54.3 for the phosphocholine group, and the two terminal methyl carbons at 13.8 and 13.9 ppm along with 9 methylene carbons in the region of 22.2–33.8 ppm. The two- and three-bond ^{31}P-^{13}C couplings and the ^{15}N-^{13}C one-bond couplings were consistent with the assignments. Similar heteronuclear coupling patterns were observed in the ^{13}C NMR spectra of two other lactone phospholipids **41** and **43**. The automatic distortionless enhancement by polarization transfer (ADEPT) spectrum of **42** (Fig. 4) was consistent with the presence of 3 methine, 12 methylene and 5 methyl carbons. The carbonyl carbons were not seen in the ADEPT spectrum. The proton assignment of the ^1H spectrum of **42** was confirmed by HETCOR (Fig. 2B). The lactone phospholipids **41** and **43**



30 R = C_6H_{13}, R^1 = OH, R_2 = H, R_3 = COC_5H_{11}, 33%

31 R = C_6H_{13}, R^1 = $OCOC_5H_{11}$, R^2 = H, R_3 = H, 70%

32 R = C_6H_{13}, R^1 = H, R^2 = $OCOC_5H_{11}$, R^3 = H, 53%

33 R = C_6H_{13}, R^1 = H, R^2 = OH, R^3 = COC_5H_{11}, 28%

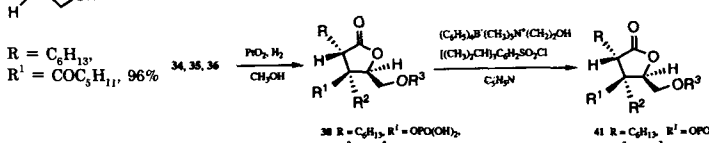
34 R = C_6H_{13}, R^1 = $OPO(OC_6H_5)_2$, R^2 = H, R^3 = COC_5H_{11}, 95%

35 R = C_6H_{13}, R^1 = $OCOC_5H_{11}$, R^2 = H, R^3 = $PO(OC_6H_5)_2$, 70%

36 R = C_6H_{13}, R^1 = H, R^2 = $OCOC_5H_{11}$, R^3 = $PO(OC_6H_5)_2$, 82%

37 R = C_6H_{13}, R^1 = COC_5H_{11}, 96%

SCHEME 9



38 R = C_6H_{13}, R^1 = $OPO(OH)_2$, R^2 = H, R^3 = COC_5H_{11}, 90%

39 R = C_6H_{13}, R^1 = $OCOC_5H_{11}$, R^2 = H, R^3 = $PO(OH)_2$, 99%

40 R = C_6H_{13}, R^1 = H, R^2 = $OCOC_5H_{11}$, R^3 = $PO(OH)_2$, 99%

41 R = C_6H_{13}, R^1 = $OPO(O^-)O(CH_2)_2N^+(CH_3)_3$, R^2 = H, R^3 = COC_5H_{11}, 36%

42 R = C_6H_{13}, R^1 = $OCOC_5H_{11}$, R^2 = H, R^3 = $PO(O^-)O(CH_2)_2N^+(CH_3)_3$, 68%

43 R = C_6H_{13}, R^1 = H, R^2 = $OCOC_5H_{11}$, R^3 = $PO(O^-)O(CH_2)_2N^+(CH_3)_3$, 43%

SCHEME 10

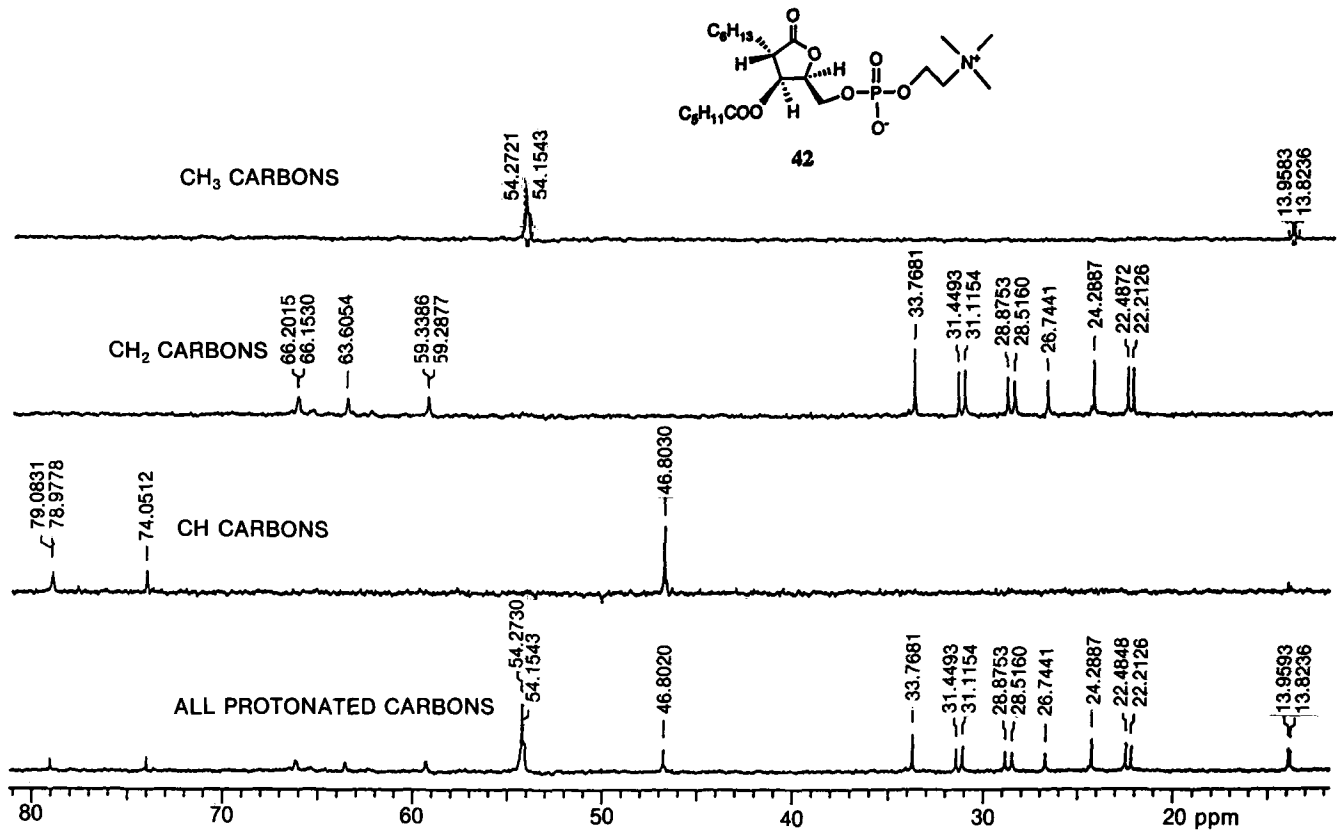


FIG. 4. The automatic distortionless enhancement by polarization transfer spectrum (75.47 MHz, chloroform-*d*) of the lactone phospholipid 42.

were assigned in a similar fashion. The stereoisomeric purity of **41, 42** and **43** was determined to be 98.2, 99.0 and 98.5% respectively, by high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

General. Unless otherwise noted, materials obtained from commercial suppliers were used without further purification. Diethyl ether (Et_2O) and THF were distilled from sodium/benzophenone immediately prior to use. Solvents for reactions, *i.e.*, acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2) and pyridine (C_5H_5N) were distilled from calcium hydride and stored over molecular sieves (4 Å) under nitrogen. All reactions except those involving water were carried out under a nitrogen atmosphere. Melting points were measured on a Thomas Hoover (Buffalo, NY) capillary melting point apparatus and are uncorrected. IR spectra were determined with a Perkin-Elmer 281B infrared spectrometer (Norwalk, CT). ^1H NMR spectra were measured at 300.13 MHz, while ^{13}C NMR were measured at 75.47 MHz on a Varian VXR-300 spectrometer (Palo Alto, CA). Solvents used were $CDCl_3$ or $CDCl_3/CD_3OD$ (3:1, vol/vol). Chemical shifts are expressed in parts per million (ppm, δ) downfield from internal tetramethylsilane. Characteristic ^1H NMR data are tabulated in the following format: multiplicity (*s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *br*, broad; *exch*, exchangeable), coupling constant(s) (*J*, in Hertz), number of protons. Low resolution mass spectra (LRMS) were obtained on a Finnigan 320 gas chromatograph/mass spectrometer (Winsford, England). HRMS were measured at the Department of Chemistry, University of Kansas (Lawrence, KS). Elemental analyses were done by Scandinavian Microanalytical Laboratory (Herlev, Denmark). Flash chromatography was carried out as described (41) on Kieselgel 60 (0.040–0.063 mm) (E. Merck, Darmstadt, Germany). TLC was done using 0.25-mm silica gel plates and Solvent A (hexane/ethyl acetate, 3:1, vol/vol) or Solvent B (methylene chloride/methanol/water, 10:10:1, by vol), or other solvent systems as specified.

Fractions were visualized by dipping the plates in a solution of ammonium molybdate (IV)tetrahydrate/water/concentrated sulfuric acid (1:9:1, wt/vol/vol) followed by heating at 115°C for 10 min, or by exposure to iodine vapors. All organic extracts were dried over anhydrous magnesium sulfate ($MgSO_4$) prior to concentration. The purity of all final and new products was checked by HPLC on a Waters Model 600E (Millipore, Milford, MA) equipped with a Waters 600E System Controller, a Waters 991 Photodiode Array Detector, a Waters automated Switching valve, a Computer NEC Power Mate SX/16 and a NEC Pinwriter P6200 (Tokyo, Japan), using a C_{18}-Nova-Pak 4.0 μm column (Waters) (Taunton, MA), or a Nova-Pak CN HP 4.0 μm column with either acetonitrile/methanol (80:20, vol/vol or acetonitrile/water (50:50, vol/vol) as mobile phase. The flow rate was 1 mL/min.

Materials. *N,N*-Dimethylacetamide, 1-bromohexane, acrolein, lithium diisopropylamide mono-tetrahydrofuran (1.5 M solution in cyclohexane, LDA), iodine (resublimed, 99.8%), triethylamine (Et_3N), DMAP, hexanoic anhydride, diphenyl chlorophosphate, platinum-(IV)oxide, 2,4,6-triisopylbenzenesulfonyl chloride, tetraphenylboron sodium and choline chloride were purchased from Aldrich (Milwaukee, WI). Silver trifluoroacetate was purchased from Spectrum Chemical MFG. Corp. (Gardena, CA).

Purification of acrolein. Acrolein was first distilled from calcium hydride into a flask that contained catechol (42) (1.0 g) in an ice bath. Anhydrous copper(II)sulfate (1.0 g) was added to this solution, which was then stirred and redistilled into a flask containing molecular sieves (4 Å) in an ice bath. Redistilled acrolein was stored in a freezer.

Preparation of choline tetraphenylborate. Sodium tetraphenylborate (6.85 g, 20.0 mmol) in water (30 mL) was added to choline chloride (4.20 g, 30.0 mmol) in the same volume of water (43). The gelatinous precipitate was filtered, washed with water (4 × 100 mL), pressed dry on the filter and then dried azeotropically with anhydrous ethanol (EtOH) and benzene (C_6H_6). Recrystallization from CH_3CN gave large hexagonal prisms (5.97 g, 65% yield), m.p. 249–251°C. IR (KBr, cm^{-1}) 3502 (*sh*), 3022, 2980, 1590, 1470, 1420, 1265, 1076, 720. ^1H NMR (pyridine-d_5) δ 2.99 (*s*, 9 H, 3 CH_3), 3.37 (*ddd, J* = 2.0, 4.1, 4.9 Hz, 2 H, CH_2), 4.1 (*br m*, 2 H, CH_2), 5.1 (*br s*, 1 H, OH), 7.12 (*dd, J* = 7.1, 7.1 Hz, 8 ArH), 7.27 (*dd, J* = 7.4, 7.5 Hz, 12 ArH). ^{13}C NMR (C_5D_5N) δ 165.9, 165.2, 164.6, 163.9, 137.0 (8 C), 126.3, 126.2, 126.2, 126.2, 122.4 (8 C), 68.8, 56.1, 54.0, 53.9, 53.9.

Preparation of N,N-*dimethyloctanamide* (**11**). To a solution of LDA (6.4 g, 59.8 mmol) in dry THF (50 mL) at -78°C was added dropwise over 3 min anhydrous *N,N*-dimethylacetamide (5.0 g, 57.4 mmol) in THF (5 mL), and the mixture was stirred at -78°C for 1 h. 1-Bromohexane (11.3 g, 68.4 mmol) in THF (5 mL) was added rapidly at -78°C and stirred for 15 min. The reaction mixture was warmed to room temperature and stirred for 1 h. The reaction was quenched with saturated aqueous NH_4Cl solution (4 mL), diluted with H_2O (50 mL), and extracted with Et_2O (3 × 100 mL). The combined organic phases were washed with brine, dried and concentrated *in vacuo*. This gave a colorless oil (9.5 g, 97% yield) after chromatography (Solvent A). Distillation at 145°C/55 mmHg afforded compound **11** (7.20 g, yield 74%); R_f = 0.25 (Solvent A). IR (neat, cm^{-1}) 2937, 1655, 1495, 1379. ^1H NMR (90 MHz) ($CDCl_3$) δ 0.92 (*dd, J* = 7.2, 7.2 Hz, 3 H, CH_3), 1.2–1.8 (*m*, 10 H, 5 CH_2), 2.32 (*dd, J* = 4.6, 6.8 Hz, 2 H), 2.94 (*s*, 3 H, NCH_3), 3.1 (*s*, 3 H, NCH_3).

N,N-*Dimethyl-2-methyl-octanamide* (**12**). This was prepared from *N,N*-dimethylpropionamide (10.4 g, 102.4 mmol): Colorless oil (18.0 g, yield 95%), R_f = 0.31 (Solvent A). IR (neat, cm^{-1}) 2940, 1660, 1485, 1380. ^1H NMR (90 MHz) ($CDCl_3$) δ 0.84–0.94 (*m*, 3 H, CH_3), 1.2–1.8 (*m*, 10 H, 5CH_2), 1.24 (*d, J* = 4.7 Hz, 3 H, CH_3), 2.46 (*m*, 1 H), 2.97 (*s*, 3 H, NCH_3), 3.0 (*s*, 3 H, NCH_3).

General procedure for the Aldol condensation. The reaction was carried out as described by Heathcock *et al.* (25). To a solution of LDA (4.22 g, 39.40 mmol) in THF (52 mL) at –20°C (ice-salt bath), was added a solution of *N,N*-dimethyloctanamide (6.15 g, 36.0 mmol) in THF (3.0 mL). The reaction mixture was stirred at –20°C for 1 h and then cooled to –78°C. Acrolein (2.41 g, 43.0 mmol) was added as rapidly as possible by means of a syringe, and the reaction mixture was stirred vigorously at –78°C for 1 h. The reaction was followed by TLC (Solvent A), which revealed two products with R_f values of 0.26 and 0.11, respectively. The reaction was quenched with saturated aqueous NH_4Cl solution (6 mL) and then warmed to room temperature. The mixture solution was diluted with water (20 mL) and extracted with Et_2O (3 × 50 mL). The combined ether extracts were washed with brine (40 mL) and dried (Na_2SO_4). Removal of the solvent *in vacuo* resulted in an oily crude residue that was purified by flash chromatography (Solvent A); it afforded *erythro-N,N*-dimethyl-3-hydroxy-2-hexyl-4-pentenamide (**6**): Colorless oil, 3.38 g, 41% yield; IR (neat, cm^{-1}) 3400 (*br*), 2920, 2850, 1640, 1620, 1490, 1395, 1260. ^1H NMR ($CDCl_3$) δ 0.82-0.90 (*m*, 3 H, CH_3), 1.17–1.27 (*m*, 8 H, 4 CH_2), 1.57–1.71 (*m*, 2 H, CH_2), 2.74 (*ddd, J* = 4.2, 4.2, 9.6 Hz, 1 H, H-2), 2.95 (*s*, 3 H, NCH_3), 3.0 (*s*, 3 H, NCH_3), 4.1 (*br s*, 1 H, OH), 4.2 (*dd, J* = 5.3, 4.2 Hz, 1 H, H-3), 5.1 (*ddd, J* = 1.5, 1.7, 10.5 Hz, 1 H, H-5A), 5.3 (*ddd, J* = 1.8, 1.8, 17.2 Hz, 1 H, H-5B), 5.8 (*ddd, J* = 5.2, 10.5, 17.3 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 176.1, 138.3, 115.4, 72.9, 45.5, 37.8, 35.5, 31.7, 29.5, 27.8, 26.8, 22.6, 13.9. LRMS *m/e* 170, 156, 143, 126, 100, 87, 72, 57. HRMS calcd. for $C_{13}H_{25}O_2N$, 227.1884; found 227.1872.

Threo-N,N-*Dimethyl-3-hydroxy-2-hexyl-4-pentenamide* (**7**). Colorless oil (2.03 g, 25% yield). IR (neat, cm^{-1}) 3630 (*sh*), 3390 (*br*), 2950, 2850, 1640, 1620, 1455, 1390, 1260. ^1H NMR ($CDCl_3$) δ 0.83–0.90 (*m*, 3 H, CH_3), 1.1–1.4 (*m*, 8 H, 4 CH_2), 1.65–1.72 (*m*, 2 H, CH_2), 2.9 (*s*, 3 H, NCH_3), 3.0 (*s*, 3 H, NCH_3), 2.75 (*ddd, J* = 7.2, 4.3, 7.3 Hz, 1 H, H-2), 4.16 (*br d, J* = 3.3 Hz, 1 H, OH), 4.38 (*br d, J* = 7.5 Hz, 1 H, H-3), 5.08 (*ddd, J* = 1.3, 1.5, 10.4 Hz, 1 H, H-5A), 5.25 (*ddd, J* = 1.5, 1.5, 17.1 Hz, 1 H H-5B), 5.80 (*ddd, J* = 5.7, 10.5, 17.1 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 175.8, 139.8, 115.2, 73.6, 45.4, 37.7, 35.2, 31.6, 30.1, 29.4, 27.3, 22.5, 14.0. LRMS *m/e* 226 (M^+ – 1), 184, 170, 157, 140, 114, 101, 72, 57. HRMS calcd. for $C_{13}H_{25}O_2N$, 227.1884; found 227.1879.

Erythro-N,N-*Dimethyl-3-hydroxy-2-methyl-2-hexyl-4-pentenamide* (**13**). Colorless oil (3.92 g, 36% yield). IR (neat, cm^{-1}) 3405 (*br*), 2920, 2860, 1645, 1620, 1495, 1395, 1260. ^1H NMR ($CDCl_3$) δ 0.83–0.89 (*dd, J* = 7.1,7.2 Hz, 3 H, CH_3), 1.20–1.36 (*m*, 9 H, 3 CH_2, CH_3), 1.52–1.80 (*m*, 2 H, CH_2), 2.22–2.40 (*m*, 4 H), 3.10 (*s*, 6 H, 2 NCH_3), 4.21 (*br s*, 1 H, OH), 5.10–5.30 (*m*, 2 H), 5.70–5.90 (*m*, 2 H). ^{13}C NMR ($CDCl_3$) δ 176.7, 139.3, 116.5, 72.9, 49.5, 37.8, 35.5, 31.7, 29.5, 27.8, 26.8, 22.6, 21.4, 13.9. LRMS *m/e* 241 (M^+), 226. HRMS calcd. for $C_{14}H_{27}O_2N$, 241.1908; found 241.1912.

Threo-N,N-*Dimethyl-3-hydroxy-2-methyl-2-hexyl-4-pentenamide* (**14**). Colorless oil (3.53 g, 33% yield). IR (neat, cm^{-1}) 3610 (*sh*), 3390 (*br*), 2960, 2840, 1645, 1625, 1455, 1396, 1270. ^1H NMR ($CDCl_3$) δ 0.84–0.90 (*m*, 3 H, CH_3), 1.21 (*s*, 3 H, CH_3), 1.24–1.32 (*m*, 6 H, 3 CH_2),

1.41–1.72 (*m*, 2 H, CH_2), 2.37 (*dd, J* = 6.9, 7.1 Hz, 2 H), 2.98 (*s*, 3 H, NCH_3), 3.02 (*s*, 3 H, NCH_3), 5.23 (*dd, J* = 1.8, 7.6 Hz, 1 H), 5.37 (*ddd, J* = 1.7, 1.9, 10.3 Hz, 1 H), 5.76–5.81 (*m*, 1 H), 5.84 (*dd, J* = 7.3, 10.4 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 176.8, 138.9, 115.0, 72.9, 48.4, 37.7, 35.2, 31.6, 30.1, 29.4, 27.3, 22.5, 20.3, 14.0. LRMS *m/e* 240 (M^+ – 1), 198, 180. HRMS calcd. for $C_{14}H_{27}O_2N$, 241.1908; found 241.1903.

General procedure for the hexanoylation of N,N-*dimethyl-3-hydroxy-2-hexyl-4-pentenamide.* To a solution of *erythro-N,N*-dimethyl-3-hydroxy-2-hexyl-4-pentenamide (**16**) (2.96 g, 13.0 mmol) in anhydrous CH_2Cl_2 (60 mL) and stirring at 0°C, hexanoic anhydride (3.35 g, 15.65 mmol), Et_3N (1.58 g, 15.65 mmol) and DMAP (0.32 g, 2.61 mmol) were added and the mixture was stirred at 0°C for 40 min (37). The reaction was monitored by TLC (Solvent A). The reaction mixture was diluted with Et_2O (100 mL) and extracted with 1.0 M H_3PO_4 (2 × 100 mL). The organic phase was washed with brine and dried over Na_2SO_4. Removal of the solvent resulted in the crude product, which was purified by flash chromatography (hexane/ethyl acetate, 4:1, vol/vol). *Erythro-N,N*-dimethyl-2-hexyl-3-[oxy(hexanoyl)]-4-pentenamide (**15**) was obtained as colorless oil (4.25 g, 98% yield). R_f = 0.46 (Solvent A). IR (neat, cm^{-1}) 2960, 2848, 1730, 1640, 1615, 1460, 1375, 1240. ^1H NMR ($CDCl_3$) δ 0.81–0.94 (*m*, 6 H, 2 CH_3), 1.04–1.40 (*m*, 12 H, 6 CH_2), 1.50–1.70 (*m*, 4 H, 2 CH_2), 2.22 (*dd, J* = 7.4, 7.5 Hz, 2 H, CH_2), 2.95–3.05 (*m*, 4 H, NCH_3 + CH), 3.11 (*s*, 3 H, NCH_3), 5.22–5.36 (*m*, 3 H), 5.75 (*ddd, J* = 7.0, 10.4, 17.4 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 172.9, 172.2, 134.1, 118.8, 76.1, 45.2, 37.6, 35.8, 34.4, 31.6, 31.2, 29.1, 28.3, 27.2, 24.5, 22.5, 22.2, 14.0, 13.8. LRMS *m/s* 325 (M^+), 297, 181, 126, 99, 87, 73, 57. HRMS calcd. for $C_{19}H_{35}O_3N$, 325.2615; found 325.2620.

Threo-N,N-*Dimethyl-2-hexyl-3-[oxy(hexanoyl)]-4-pentenamide* (**16**). Colorless oil (4.60 g, 95% yield). R_f = 0.37 (Solvent A). IR (neat, cm^{-1}) 2920, 2840, 1735, 1640, 1620, 1460, 1395, 1240. ^1H NMR ($CDCl_3$) δ 0.81–0.89 (*m*, 6 H, 2 CH_3), 1.05–1.40 (*m*, 12 H, 6 CH_2), 1.50–1.65 (*m*, 4 H, 2 CH_2), 2.30 (*dd, J* = 7.4, 7.5 Hz, 2 H, CH_2), 2.95–3.04 (*m*, 4 H, NCH_3 + CH), 3.11 (*s*, 3 H, NCH_3), 5.20–5.36 (*m*, 3 H), 5.76 (*ddd, J* = 7.0, 10.0, 17.1 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 172.8, 172.2, 134.1, 118.9, 76.1, 45.1, 37.5, 35.8, 34.4, 27.2, 31.6, 31.1, 29.3, 28.3, 24.5, 22.5, 22.2, 14.0, 13.8. LRMS *m/s* 325 (M^+), 297, 181, 126, 99, 87, 73, 57. HRMS calcd. for $C_{19}H_{35}O_3N$, 325.2165; found 325.2167.

Erythro-N,N-*Dimethyl-2-methyl-2-hexyl-3-[oxy(hexanoyl)]-4-pentenamide* (**17**). Colorless oil (0.56 g, 92% yield). R_f = 0.46 (Solvent A). IR (neat, cm^{-1}) 2945, 2835, 1730, 1645, 1615, 1475, 1370, 1230. ^1H NMR ($CDCl_3$) δ 0.84–0.96 (*m*, 6 H, 2 CH_3), 1.24 (*s*, 3 H, CH_3), 1.26–1.42 (*m*, 8 H, 4 CH_2), 1.56–1.70 (*m*, 4 H, 2 CH_2), 2.34 (*dd, J* = 7.4, 7.5 Hz, 2 H, CH_2), 3.06 (*br s*, 6 H, 2 NCH_3), 5.10–5.18 (*m*, 1 H), 5.18 (*d, J* = 1.2 Hz, 1 H), 5.71–5.81 (*m*, 1 H), 5.85 (*dd, J* = 4.9, 5.2 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 180.1, 173.3, 133.7, 117.2, 75.8, 50.9, 38.2, 36.2, 34.4, 34.0, 31.6, 31.2, 29.8, 24.7, 24.3, 22.6, 22.3, 19.3, 13.9, 13.8. LRMS *m/s* 339 (M^+), 311, 181, 126, 99. HRMS calcd. for $C_{20}H_{37}O_3N$, 339.2771; found 339.2779.

Threo-N,N-*Dimethyl-2-methyl-2-hexyl-3-[oxy(hexanoyl)]-4-pentenamide* (**18**). Colorless oil (0.54 g, 90% yield). R_f = 0.37 (Solvent A). IR (neat, cm^{-1}) 2930, 2845, 1740, 1635, 1610, 1475, 1395, 1230. ^1H NMR ($CDCl_3$) δ 0.83–0.91 (*m*, 6 H, 2 CH_3), 1.20–1.40 (*m*, 13 H, 5 CH_2 + CH_3), 1.52–1.74 (*m*, 4 H, 2 CH_2), 2.26 (*dd, J* = 7.2, 7.4 Hz, 2 H, CH_2), 2.32 (*dd, J* = 7.3, 7.5 Hz, 2 H, CH_2), 3.02 (*br s*, 6 H, 2 NCH_3), 5.22 (*dd, J* = 1.5, 6.6 Hz, 1 H), 5.27 (*ddd, J* = 1.7, 1.9, 9.7 Hz, 1 H), 5.72–5.78 (*m*, 1 H), 5.81 (*dd, J* = 6.4, 10.2 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 180.1, 173.3, 133.7, 117.2, 75.8, 50.9, 38.2, 36.2, 34.4, 34.0, 31.6, 31.2, 29.8, 24.7, 24.3, 22.6, 22.3, 19.3, 13.9, 13.8. LRMS *m/e* 339 (M^+). HRMS calcd. for $C_{20}H_{37}O_3N$, 339.2771; found 339.2764.

General procedure for iodolactonization. To a solution of *erythro-N,N*-dimethyl-2-hexyl-3-[oxy(hexanoyl)]-4-pentenamide (**15**) (4.37 g, 12.40 mmol) in THF (100 mL), iodine (4.72 g, 18.60 mmol) and water (4.84 mL, 268.67 mmol) were added. The flask was wrapped with aluminum foil and stirred at room temperature (30). The reaction was followed by TLC (hexane/ethyl acetate, 3:2, vol/vol). After the starting material had disappeared (12–24 h), the reaction mixture was diluted with Et_2O (200 mL), washed with 5% sodium sulfite solution (3 × 150 mL) until the ether layer was colorless. Then the ether layer was washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography (hexane/ethyl acetate, 3:2, vol/vol) which afforded (3α, 4β, 5β)dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**23**) as pale oil (4.90 g, 86% yield). R_f = 0.75 (hexane/ethyl acetate, 3:2, vol/vol). IR (neat, cm^{-1}) 2960, 2860, 1785, 1740, 1460, 1240. ^1H NMR ($CDCl_3$) δ 0.86–0.92 (*m*, 6 H, 2 CH_3), 1.21–1.34 (*m*, 10 H, 5 CH_2), 1.46–1.55 (*m*, 2 H CH_2), 1.58–1.80 (*m*, 4 H, 2 CH_2), 2.36 (*ddd, J* = 1.5, 7.2, 7.3 Hz, 2 H, CH_2), 2.63 (*ddd, J* = 1.3, 7.5, 7.5 Hz, 1 H, H-3), 3.32 (*ABq, J* = 8.6, 9.8 Hz, 1 H, H-6A), 3.38 (*ABq, J* = 5.6, 9.4 Hz, 1 H, H-6B), 4.76 (*ddd, J* = 5.9, 4.5, 8.8 Hz, 1 H, H-5), 5.28 (*dd, J* = 1.5, 4.5 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 176.1, 172.5, 79.5, 74.2, 48.7, 34.4, 26.9, 31.4, 31.2, 28.8, 28.5, 24.4, 22.5, 22.3, 14.0, 13.8, -2.6. LRMS *m/e* 297 (M^+ – I), 241, 213, 198, 100, 84, 71, 57. HRMS calcd. for $C_{17}H_{29}O_4$ (M^+ – I), 297.2065; found 297.2059.

(3α,4β,5β)Dihydro-4-hydroxy-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**19**). Pale oil (0.07 g, 60% yield). R_f = 0.62 (Solvent A). IR (neat, cm^{-1}) 3520–3220 (*br*), 2920, 2860, 1730, 1455, 1230. ^1H NMR ($CDCl_3$) δ 0.85–0.91 (*m*, 3 H, CH_3), 1.25–1.38 (*m*, 1 H, CH_2), 1.42–1.53 (*m*, 2 H, CH_2), 1.55–1.62 (*m*, 1 H), 1.65–1.74 (*m*, 1 H), 2.14 (*s*, 3 H, CH_3), 2.21 (*d, J* = 5.0 Hz, 1 H, OH), 2.65 (*ddd, J* = 1.5, 7.4, 7.5 Hz, 1 H, H-3), 3.39–3.44 (*m*, 2 H, H-6), 4.40 (*dd, J* = 1.5, 4.5 Hz, 1 H, H-4), 4.76 (*ddd, J* = 6.0, 4.5, 8.7 Hz, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 176.0, 79.4, 74.4, 48.6, 31.4, 28.8, 26.9, 22.5, 20.7, 14.0, -2.6.

(3α,4β,5α)Dihydro-4-hydroxy-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**20**). 0.01 g, 8% yield, m.p. 82–84°C, R_f = 0.68 (Solvent A). IR (neat, cm^{-1}) 3520 (*sh*), 3420 (*br*), 2950, 2850, 1730, 1455, 1215. ^1H NMR ($CDCl_3$) δ 0.85–0.92 (*m*, 3 H, CH_3), 1.20–1.50 (*m*, 8 H, 4 CH_2), 1.52–1.74 (*m*, 1 H), 1.74–1.85 (*m*, 1 H), 1.96 (*br s*, 1 H,

OH), 2.60 (*ddd, J* = 4.8, 4.8, 9.9 Hz, 1 H, H-3), 3.30–3.50 (*m*, 2 H, H-6), 4.50 (*ddd, J* = 3.0, 5.7, 9.7 Hz, 1 H, H-5), 4.63 (*br d, J* = 3.3 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 176.8, 81.1, 69.7, 47.2, 31.5, 29.6, 27.5, 23.3, 22.6, 14.0, 1.0.

(3α,4α,5β)Dihydro-4-hydroxy-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**21**). Pale oil (0.10 g, 63% yield). R_f = 0.67 (Solvent A). IR (neat, cm^{-1}) 3540 (*sh*), 2940, 2850, 1760, 1440, 1220. ^{1}H NMR ($CDCl_3$) δ 0.82–0.92 (*m*, 3 H, CH_3), 1.12–1.56 (*m*, 8 H, 4 CH_2), 1.58–1.74 (*m*, 1 H), 1.79–1.90 (*m*, 1 H), 1.98 (*d, J* = 4.8 Hz, 1 H, OH), 2.60 (*ddd, J* = 8.4, 5.2, 5.2 Hz, 1 H, H-3), 3.36–3.47 (*m*, 2H, H-6), 4.49 (*ddd, J* = 3.2, 6.1, 9.1 Hz, 1 H, H-5), 4.63 (*ddd, J* = 3.6, 5.2, 5.2 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 178.6, 83.2, 74.2, 49.4, 34.5, 32.1, 26.8, 25.6, 23.5, 17.1, -1.2.

(3α,4α,5α)Dihydro-4-hydroxy-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**22**). 0.03 g, 11% yield, m.p. 66–68°C, R_f = 0.58 (Solvent A). IR (neat, cm^{-1}) 3520 (*sh*), 2910, 2850, 1760, 1440, 1220, 1170. ^{1}H NMR ($CDCl_3$) δ 0.82–0.93 (*m*, 3 H, CH_3), 1.20–1.54 (*m*, 8 H, 4 CH_2), 1.60–1.85 (*m*, 2 H, CH_2), 2.16–2.35 (*br s*, 1 H, OH), 2.62–2.71 (*m*, 1 H, H-3), 3.17 (*ABq, J* = 8.5, 10.7 Hz, 1 H, H-6A), 3.36 (*ABq, J* = 4.8, 11.0 Hz, 1 H, H-6B), 4.44–4.52 (*m*, 2 H). ^{13}C NMR ($CDCl_3$) δ 176.7, 84.6, 72.3, 44.1, 31.5, 29.6, 27.6, 23.6, 22.5, 14.0, 2.1.

(3α,4α,5β)Dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**24**). 2.79 g, 59% yield, R_f = 0.66 (Solvent A), m.p. 60–62°C. IR (KBr, cm^{-1}) 2960, 2860, 1785, 1740, 1465, 1240. ^{1}H NMR ($CDCl_3$) δ 0.86–0.92 (*m*, 6 H, 2 CH_3), 1.20–1.44 (*m*, 12 H, 6 CH_2), 1.46–1.70 (*m*, 3 H, CH_2), 1.71–1.90 (*m*, 1 H), 2.31–2.57 (*m*, 2 H, CH_2), 2.91 (*ddd, J* = 5.3, 7.0, 9.2 Hz, 1 H, H-3), 3.23–3.36 (*m*, 2 H, H-6), 4.40 (*ddd, J* = 1.2, 5.8, 5.9 Hz, 1 H, H-5), 5.31 (*dd, J* = 1.2, 6.9 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 176.0, 172.8, 82.3, 73.6, 43.5, 33.9, 31.2, 31.8, 29.7, 27.4, 24.5, 24.4, 23.4, 22.2, 14.1, 13.9, -2.2. LRMS *m/e* 424 (M^+, weak), 368, 325, 296 (M^+ – H-I), 225, 181, 99, 71, 55. HRMS calcd. for $C_{17}H_{29}O_4$ (M^+ – I), 297.2065; found 297.2077.

(3α,4α,5α)Dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-hexyl-2(3H)-furanone (**25**). 1.42 g, 30% yield, R_f = 0.65 (Solvent A), m.p. 60–62°C. IR (KBr, cm^{-1}) 2960, 2860, 1785, 1740, 1465, 1240. ^{1}H NMR ($CDCl_3$) δ 0.80–0.98 (*m*, 6 H, 2 CH_3), 1.18–1.44 (*m*, 12 H, 6 CH_2), 1.45–1.71 (*m*, 3 H), 1.72–1.89 (*m*, 1 H), 2.37 (*dd, J* = 8.2, 6.9 Hz, 2 H, CH_2), 2.75 (*ddd, J* = 3.6, 4.4, 8.1 Hz, 1 H, H-3), 3.21–3.34 (*m*, 2 H, H-6), 4.63 (*ddd, J* = 3.4, 6.6, 9.0 Hz, 1 H, H-5), 5.84 (*dd, J* = 3.3, 5.1 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 175.6, 172.2, 80.2, 70.7, 46.4, 33.3, 31.4, 31.2, 29.0, 27.2, 24.4, 24.1, 23.7, 22.2, 13.9, 13.8, -3.0. LRMS *m/e* 424 (M^+), 368, 325, 296 (M^+ – H – I), 225, 181, 99, 71, 55, HRMS calcd. for $C_{17}H_{29}O_4$ (M^+ – I), 297.2065; found 297.2059.

(3α,4α,5α)Dihydro-4-hydroxy-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**26**). 0.14 g, 98% yield, m.p. 72–74°C, R_f = 0.51 (Solvent A). IR (neat, cm^{-1}) 3600, 3420, 2920, 2845, 1770, 1490, 1270. ^{1}H NMR ($CDCl_3$) δ 0.88 (*dd, J* = 6.5, 6.7 Hz, 3 H, CH_3), 1.23 (*s*, 3 H, CH_3), 1.24–1.70 (*m*, 6 H, 3 CH_2), 1.71–1.92 (*m*, 4 H, 2 CH_2), 2.18–2.32 (*br d, J* = 2.6 Hz, 1 H, OH), 3.34–3.44 (*m*, 2 H, H-6), 4.21 (*br d, J* = 3.3 Hz, 1 H, H-4), 4.66 (*ddd, J* = 3.2, 6.1, 9.3 Hz, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 180.2, 79.9, 75.1, 48.8, 31.6, 30.6, 29.7, 23.6, 22.6, 19.6, 13.9, 0.88. HRMS calcd. for $C_{12}H_{21}O_3$ (M^+ – I), 213.2961; found 213.2954.

(3α,4α,5α)Dihydro-4-hydroxy-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**27**). 0.13 g, 86% yield, m.p. 71–73°C, R_f = 0.51 (Solvent A). IR (neat, cm^{-1}) 3600, 3420, 2920, 2845, 1770, 1490, 1270. ^{1}H NMR ($CDCl_3$) δ 0.88 (*dd, J* = 6.5, 6.7 Hz, 3 H, CH_3), 1.23 (*s*, 3 H, CH_3), 1.24–1.70 (*m*, 6 H, 3 CH_2), 1.71–1.92 (*m*, 4 H, 2 CH_2), 2.18–2.32 (*br d, J* = 2.6 Hz, 1 H, OH), 3.34–3.44 (*m*, 2 H, H-6), 4.21 (*br d, J* = 3.3 Hz, 1 H, H-4), 4.66 (*ddd, J* = 3.2, 6.1, 9.3 Hz, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 180.6, 79.7, 75.4, 48.9, 31.6, 30.6, 29.7, 23.6, 22.6, 19.2, 14.0, 0.88. HRMS calcd. for $C_{12}H_{21}O_3$ (M^+ – I), 213.2961; found 212.2967.

(3α,4β,5β)Dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**28**). Pale oil (2.19 g, 81% yield). R_f = 0.68 (Solvent A). IR (neat, cm^{-1}) 2920, 2845, 1780, 1740, 1460, 1180. ^{1}H NMR ($CDCl_3$) δ 0.84–0.92 (*m*, 6 H, 2 CH_3), 1.10 (*s*, 3 H, CH_3), 1.20–1.45 (*m*, 13 H, CH_2), 1.55–1.70 (*m*, 5 H, CH_2), 2.37 (*ddd, J* = 2.8, 7.6, 7.6 Hz, 2 H, CH_2), 3.18 (*ABq, J* = 6.4, 10.2 Hz, 1 H, H-6A), 3.27 (*ABq, J* = 8.4, 10.4 Hz, 1 H, H-6B), 4.78 (*ddd, J* = 3.72, 6.4, 8.5 Hz, 1 H, H-5), 5.47 (*d, J* = 3.7 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 178.6, 172.1, 79.0, 75.5, 48.3, 33.9, 31.5, 31.3, 30.9, 29.8, 24.4, 23.3, 22.5, 22.2, 19.4, 14.0, 13.8, -0.88. LRMS *m/e* 311 (M^+ – I), 239, 195, 169, 129, 99, 71, 57. *Anal.* calcd. for $C_{18}H_{31}O_4I$: C, 49.32; H, 7.13; I, 28.9. Found: C, 49.36, H, 7.19, I, 28.97. HRMS calcd. for $C_{18}H_{31}O_4$ (M^+ – I), 311.2222; found 311.2166.

(3α,4α,5α)Dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**29**). Pale oil (0.73 g, 70% yield), R_f = 0.57 (Solvent A). IR (neat, cm^{-1}) 2960, 2840, 1770, 1740, 1460, 1200. ^{1}H NMR ($CDCl_3$) δ 0.83–0.92 (*m*, 6 H, 2 CH_3), 1.22–1.35 (*m*, 17 H, 7 CH_2 + CH_3), 1.46–1.50 (*m*, 1 H), 1.60–1.70 (*m*, 3 H), 2.35 (*ddd, J* = 1.7, 7.5, 7.5 Hz, 2 H, 1′-CH_2), 3.27 (*ddd, J* = 5.7, 9.6, 9.7 Hz, 2 H, H-6), 4.77 (*ddd, J* = 4.1, 5.6, 9.7 Hz, 1 H, H-5), 5.46 (*d, J* = 4.1 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 178.6, 172.4, 78.4, 75.2, 49.0, 35.6, 33.8, 31.4, 31.2, 29.4, 24.4, 23.9, 22.5, 22.2, 15.5, 14.0, 13.8, -2.6. HRMS calcd. for $C_{18}H_{31}O_4$ (M^+ – I), 311.2222; found 311.2216.

(3α,4α,5β)Dihydro-4-[oxy(hexanoyl)]-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**44**). Pale oil (0.18 g, 16% yield). R_f = 0.50 (Solvent A). IR (neat, cm^{-1}) 2920, 2850, 1780, 1740, 1460, 1350, 1180. ^{1}H NMR ($CDCl_3$) δ 0.83–0.94 (*m*, 6 H, 2 CH_3), 1.14–1.38 (*m*, 15 H, 6 CH_2 + CH_3), 1.41–1.52 (*m*, 2 H, CH_2), 1.55–1.72 (*m*, 4 H, 2 CH_2), 2.35 (*ddd, J* = 2.8, 7.6, 7.5 Hz, 2 H, CH_2), 3.24 (*ddd, J* = 6.4, 8.4, 10.2 Hz, 2 H, H-6), 4.77 (*ddd, J* = 3.7, 6.4, 8.5 Hz, 1 H, H-5), 5.45 (*d, J* = 3.7 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 178.6, 172.1, 79.0, 75.5, 48.3, 33.9, 31.5, 31.3, 30.9, 29.8, 24.4, 23.3, 22.5, 22.2, 19.5, 14.0, 13.8, -2.8. HRMS calcd. for $C_{18}H_{31}O_4$ (M^+ – I), 311.2222; found 311.2229.

(3α,4α,5β)Dihydro-4-hydroxy-5-(iodomethyl)-3-methyl-3-hexyl-2(3H)-furanone (**45**). Pale oil (0.02 g, 9% yield), R_f = 0.46 (Solvent A). IR (neat, cm^{-1}) 3610, 3412, 2940, 2845, 1780, 1490, 1210. ^{1}H NMR ($CDCl_3$) δ 0.86–0.96 (*m*, 3 H, CH_3), 1.24–1.50 (*s*, 11 H, 4 CH_2 + CH_3), 1.51–1.70 (*m*, 2 H, CH_2), 2.07 (*br d, J* = 4.3 Hz, 1 H, OH), 3.41–3.49 (*m*, 2 H, H-6), 4.29 (*dd, J* = 3.2, 3.5

Hz, 1 H, H-4), 4.76 (*ddd, J* = 3.6, 4.2, 6.1 Hz, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 179.6, 78.7, 74.8, 49.6, 35.6, 31.5, 29.5, 24.0, 22.5, 15.2, 14.0, -0.77. HRMS calcd. for $C_{12}H_{21}O_3$ ($M^+ - I$), 213.2961; found 213.2955.

General procedure for the conversion of iodolactones to hydroxy lactones. Preparation of (3α, 4β, 5β)dihydro-4-[oxy(hexanoyl)]-5-(hydroxymethyl)-3-hexyl-2(3H)-furanone (**31**). Silver trifluoroacetate (3.26 g, 14.75 mmol) was added in one portion to a stirred solution of iodolactone **23** (4.17 g, 9.84 mmol) and 1 equivalent of water (0.18 g) in nitromethane (100 mL) at 60°C. During 1–3 h, the formation of insoluble silver iodide was observed (33). After all the starting material had disappeared (20 h), as judged by TLC (Solvent A), the reaction mixture was filtered through Celite and concentrated. Flash chromatography on silica gel (Solvent A) afforded compounds **31** (2.17 g, 70% yield) and **33** (0.93 g, 28% yield). Compound **31**: white waxy solid, R_f = 0.22 (Solvent A), m.p. 42–43°C. IR (KBr, cm^{-1}) 3620–3300 (*br*), 2960, 2840, 1760, 1740, 1445, 1250, 1170. ^1H NMR ($CDCl_3$) δ 0.84–0.91 (*m*, 6 H, 2 CH_3), 1.20–1.32 (*m*, 10 H, 5 CH_2), 1.40–1.78 (*m*, 6 H, 3 CH_2), 2.34 (*dd, J* = 7.4, 7.4 Hz, 2 H, CH_2), 2.60 (*ddd, J* = 3.6, 7.6, 8.0 Hz, 1 H, H-3), 4.28–4.34 (*m*, 1 H, H-4), 4.44 (*ddd, J* = 12.4, 9.9, 5.2 Hz, 2 H, H-6), 4.51-4.60 (*m*, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 177.3, 174.2, 79.0, 72.7, 61.6, 48.9, 34.0, 31.5, 31.2, 28.9, 28.6, 26.9, 24.4, 22.5, 22.2, 13.9, 13.8. LRMS *m/e* 314 (M^+), 271, 230, 185, 155, 142, 127, 98, 57. *Anal.* calcd. for $C_{17}H_{30}O_5$: C, 64.94; H, 9.62. Found: C, 65.08; H, 9.77.

(3α,4α,5β)Dihydro-4-hydroxy-5-[methyleneoxy(hexanoyl)]-3-hexyl-2(3H)-furanone (**30**). White solid (0.82 g, 33% yield). R_f = 0.30 (Solvent A), m.p. 62–64°C. IR (KBr, cm^{-1}) 3580–3100 (*br*), 2950, 2860, 1765, 1735, 1460, 1375, 1250, 1160. ^1H NMR ($CDCl_3$) δ 0.85–0.91 (*m*, 6 H, 2 CH_3), 1.20–1.48 (*m*, 12 H, 6 CH_2), 1.58–1.87 (*m*, 4 H, 2 CH_2), 2.33 (*ddd, J* = 7.4, 7.2, 7.1 Hz, 2H, CH_2), 2.73 (*ddd, J* = 4.8, 4.8, 9.8 Hz, 1 H, H-3), 3.9 (*br s*, 1 H, OH), 4.60 (*dd, J* = 5.8, 11.9 Hz, 1 H, H-4), 4.36–4.43 (*m*, 2 H, H-6), 4.27 (*ddd, J* = 1.1, 5.8, 11.8 Hz, 1 H, H-5). ^{13}C NMR ($CDCl_3$) δ 176.9, 174.6, 79.6, 69.2, 61.2, 46.6, 34.0, 31.5, 31.2, 29.1, 27.4, 24.5, 23.1, 22.5, 22.2, 14.0, 13.8. *Anal.* calcd. for $C_{17}H_{30}O_5$: C, 64.94; H, 9.62. Found: C, 64.81; H, 9.53.

(3α,4α,5β)Dihydro-4-[oxy(hexanoyl)]-5-(hydroxymethyl)-3-hexyl-2(3H)-furanone (**32**). Pale oil (1.29 g, 53% yield). R_f = 0.18 (Solvent A). IR (neat, cm^{-1}) 3640–3200 (*br*), 2960, 2860, 1780, 1735, 1460, 1240, 1160. ^1H NMR ($CDCl_3$) δ 0.82–0.91 (*m*, 6 H, 2 CH_3), 1.20–1.40 (*m*, 12 H, 6 CH_2), 1.55–1.67 (*m*, 3 H), 1.70–1.82 (*m*, 1 H), 2.33 (*ddd, J* = 2.6, 7.5, 7.5 Hz, 2 H, CH_2), 2.95 (*ddd, J* = 6.8, 5.2, 9.7 Hz, 1 H, H-3), 3.91 (*dd, J* = 3.0, 3.0 Hz, 2 H, H-6), 4.34 (*dd, J* = 3.0, 3.2 Hz, 1 H, H-5), 5.42 (*d, J* = 6.8 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 177.9, 173.3, 84.6, 72.6, 62.0, 43.2, 34.1, 31.5, 31.2, 29.1, 27.5, 24.5, 24.1, 22.5, 22.2, 14.0, 13.8. LRMS *m/e* 314 (M^+), 283, 217, 199, 187, 99, 79, 57. *Anal.* calcd. for $C_{17}H_{30}O_5$: C, 64.94; H, 9.62. Found: C, 64.87; H, 9.47.

(3α,4β,5β)Dihydro-4-hydroxy-5-[methyleneoxy(hexanoyl)]-3-hexyl-2(3H)-furanone (**33**). Pale oil, R_f = 0.32 (Solvent A). IR (neat, cm^{-1}) 3640–3200 (*br*), 2960, 2680, 1775, 1740, 1450, 1250, 1160. ^1H NMR ($CDCl_3$) δ 0.80–0.95 (*m*, 6 H, 2 CH_3), 1.20–1.38 (*m*, 10 H, 5 CH_2), 1.40–1.50 (*m*, 2 H, CH_2), 1.56–1.76 (*m*, 3 H), 1.71–1.83 (*m*, 1 H), 2.10–2.30 (*br s*, 1 H, OH), 2.35 (*dd, J* = 7.4, 7.6 Hz, 2 H, CH_2), 2.74 (*ddd, J* = 4.4, 6.1, 8.3 Hz, 1 H, H-3), 3.75–3.83 (*m*, 2 H, H-6), 4.68 (*ddd, J* = 5.1, 5.2, 5.2 Hz, 1 H, H-5), 5.27 (*dd, J* = 5.6, 4.4 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 176.2, 173.3, 79.6, 74.3, 60.3, 46.4, 33.9, 31.4, 31.2, 28.9, 28.7, 26.7, 24.4, 22.5, 22.2, 14.0, 13.8. LRMS *m/e* 314 (M^+), 283, 217, 199, 187, 168, 131, 115, 99, 85, 57. *Anal.* calcd. for $C_{17}H_{30}O_5$: C, 64.94; H, 9.62. Found: C, 64.67; H, 9.84.

General procedure for the preparation of diphenyl phosphoric acid esters of hydroxy lactones. Phosphoric acid, (3α,4α,5β)dihydro-4-[oxy(hexanoyl)]-3-hexyl-2(3H)-furanone-5-[methyleneoxy-(diphenyl)] ester (**36**). The hydroxy lactone **32** (1.0 g, 3.19 mmol) was dissolved in dry pyridine (20 mL) in a 100-mL, tightly stoppered flask with a stir bar. Diphenyl chlorophosphate (2.44 g, 9.08 mmol) in C_5H_5N (6 mL) was added, and the mixture was stirred at room temperature for 3 h (18). The reaction was followed by TLC (Solvent A). Water (4 mL) was added, and then the mixture was concentrated to a small volume *in vacuo*. The residue was added to a mixture of water and Et_2O. The organic phase was separated, and the aqueous phase was extracted with Et_2O (2 × 40 mL). The ether extracts were combined, and concentrated *in vacuo*, and the residue was purified on silica gel (Solvent A) to yield **36** (1.42 g, 82% yield): Pale yellowish oil, R_f = 0.51 (Solvent A). IR (neat, cm^{-1}) 2960, 2860, 1780, 1736, 1588, 1482, 1350, 1288, 1180. ^1H NMR ($CDCl_3$) δ 0.81–0.90 (*m*, 6 H, 2 CH_3) 1.18–1.38 (*m*, 13 H, CH_2), 1.70–1.88 (*m*, 3 H, CH_2), 2.33 (*ddd, J* = 3.5, 7.4, 7.5 Hz, 2 H, CH_2), 2.57 (*ddd, J* = 6.9, 5.2, 9.7 Hz, 1 H, H-3), 5.30 (*d, J* = 6.9 Hz, 1 H, H-4), 4.49–4.53 (*ddd, J* = 2.6, 2.8, 5.5 Hz, 2 H, H-6), 4.43–4.46 (*m*, 1 H, H-5), 7.30–7.40 (*m*, 4 ArH), 7.16–7.24 (*m*, 6 ArH). ^{13}C NMR ($CDCl_3$) δ 176.1, 173.1, 150.2, 150.1, 129.9 (4 C), 124.9 (2 C), 119.9 (2 C), 119.8 (2 C), 81.4, 71.7, 67.4, 42.6, 33.9, 31.5, 31.1, 29.1, 27.5, 24.4, 24.1, 22.5, 22.2, 13.9, 13.8. HRMS calcd. for $C_{29}H_{39}O_8P$, 546.5931; found 546.5928. *Anal.* calcd. for $C_{29}H_{39}O_8P$: C, 63.73; H, 7.19; P, 5.67. Found: C, 63.62; H, 7.31; P, 5.77.

Phosphoric acid, (3α,4β,5β)dihydro-5-[methyleneoxy-(hexanoyl)]-3-hexyl-2(3H)-furanone-4-[oxy(diphenyl)] ester (**34**). Colorless oil (1.56 g, 95% yield). R_f = 0.63 (Solvent A). IR (neat, cm^{-1}) 2960, 2860, 1780, 1725, 1590, 1480, 1350, 1280, 1185, 1110. ^1H NMR ($CDCl_3$) δ 0.82–0.90 (*m*, 6 H, 2 CH_3), 1.17–1.44 (*m*, 12 H, 6 CH_2), 1.44–1.78 (*m*, 4 H, 2 CH_2), 2.27 (*dd, J* = 7.0, 7.4 Hz, 2 H, CH_2), 2.76 (*m*, 1 H, H-3), 4.73 (*ddd, J* = 1.7, 4.6, 10.2 Hz, 1 H, H-4), 4.25–4.36 (*m*, 2 H, H-6), 5.13 (*ddd, J* = 3.1, 5.6, 6.9 Hz, 1 H, H-5), 7.14–7.22 (*m*, 4 ArH), 7.33–7.38 (*m*, 6 ArH). ^{13}C NMR ($CDCl_3$) δ 175.0, 173.0, 149.9, (2 C), 129.9 (4 C), 125.8, 125.9, 120.0, 119.9, 119.87, 119.81, 79.0, 76.6, 61.2, 47.7, 33.9, 31.3, 31.1, 28.7, 28.1, 26.6, 24.4, 22.4, 22.2, 14.0, 13.8. HRMS calcd. for $C_{29}H_{39}O_8P$, 546.5931; found 546.5924. *Anal.* calcd. for $C_{29}H_{39}O_8P$: C, 63.73; H, 7.19; P, 5.67. Found: C, 63.84; H, 7.36; P, 5.81.

Phosphoric acid, (3α,4β,5β)dihydro-4-[oxy(hexanoyl)]-3-hexyl-2(3H)-furanone-5-[methyleneoxy(diphenyl)] ester

(**35**). Colorless oil (0.73 g, 70% yield). R_f = 0.52 (Solvent A). IR (neat, cm^{-1}) 2960, 2855, 1774, 1735, 1588, 1460, 1350, 1290, 1085. ^1H NMR ($CDCl_3$) δ 0.82–0.92 (*m*, 6 H, 2 CH_3), 1.20–1.44 (*m*, 12 H, 6 CH_2), 1.50–1.72 (*m*, 3 H), 1.78–1.80 (*m*, 1 H), 2.29 (*dd, J* = 7.3, 7.6 Hz, 2 H, CH_2), 2.60 (*ddd, J* = 6.0, 4.7, 8.7 Hz, 1 H, H-3), 5.22 (*dd, J* = 4.6, 5.9 Hz, 1 H, H-4), 4.36–4.49 (*m*, 2 H, H-6), 4.76 (*dddd, J* = 1.3, 5.2, 5.1, 5.1 Hz, 1 H, H-5), 7.30–7.44 (*m*, 4 ArH), 7.18–7.20 (*m*, 6 ArH). ^{13}C NMR ($CDCl_3$) δ 175.2, 172.9, 150.2 (2 C), 129.9 (4 C), 125.6 (2 C), 119.9 (4 C), 79.4, 73.7, 65.7, 46.0, 33.4, 31.34, 31.1, 28.8, 28.6, 26.6, 24.3, 22.5, 22.2, 13.9, 13.8. HRMS calcd. for $C_{29}H_{39}O_8P$, 546.5931; found 546.5929. *Anal.* calcd. for $C_{29}H_{39}O_8P$: C, 63.73; H, 7.19; P, 5.67. Found: C, 63.85; H, 7.28; P, 5.81.

3-Hexyl-5-[methyleneoxy(hexanoyl)]-2(5H)-furanone (**37**). Colorless oil (0.33 g, 96% yield, R_f = 0.61 (Solvent A). IR (neat, cm^{-1}) 2960, 2850, 1760, 1740, 1650, 1455, 1250, 1160, 1050. ^1H NMR ($CDCl_3$) δ 0.84–0.90 (*m*, 6 H, 2 CH_3), 1.20–1.38 (*m*, 10 H, 5 CH_2), 1.47–1.62 (*m*, 4 H, 2 CH_2), 2.20–2.32 (*m*, 4 H, 2 CH_2), 2.27 (*m*, 4 H, 2 CH_2), 4.23 (*ABq, J* = 3.8, 12.0 Hz, 1 H, H-6A), 4.31 (*ABq, J* = 5.2, 12.0 Hz, 1 H, H-6B), 5.07 (*ddd, J* = 1.7, 3.1, 7.5 Hz, 1 H, H-5), 6.93 (*d, J* = 1.7 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3$) δ 173.3, 173.0, 143.5, 136.6, 78.7, 62.8, 33.9, 31.4, 31.2, 28.9, 28.1, 27.3, 24.5, 22.4, 22.2, 13.9, 13.8. HRMS calcd. for $C_{17}H_{28}O_4$, 296.4058; found 296.4041. *Anal.* calcd. for $C_{17}H_{28}O_4$:C, 68.89; H, 9.52. Found: C, 68.76; H, 9.43.

General procedure for the hydrogenolysis of diphenyl phosphoric acid esters. Phosphoric acid, (3α,4α,5β)dihydro-4-[oxy(hexanoyl)]-3-hexyl-2(3H)-furanone-5-(methyleneoxy) ester (**40**). The phosphoric acid diphenyl ester (**36**) (1.37 g, 2.51 mmol) in CH_3OH (50 mL) was mixed with PtO_2 (0.29 g, 1.25 mmol) in an 100-mL flask attached to a balloon filled with hydrogen (18). The reaction was stirred at room temperature, and the reaction progress was monitored by TLC (hexane/ethyl acetate, 1:1, vol/vol) to detect the loss of the starting material and, with methanol as solvent, to detect the much slower loss of the monophenyl ester. The reaction time varied, but averaged about 12–24 h. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo*. The product **40** (1.03 g, 99% yield) was directly used without further purification: Colorless oil, R_f = 0.70 (Solvent B). IR (neat, cm^{-1}) 3640–3000 (*br*), 2962, 2860, 1780, 1720, 1460, 1355, 1150, 980. ^1H NMR ($CDCl_3$) δ 0.84–0.93 (*m*, 6 H, 2 CH_3), 1.20–1.76 (*m*, 16 H, 8 CH_2), 2.32 (*dd, J* = 7.5, 7.6 Hz, 2 H, CH_2), 2.90-3.0 (*m*, 1 H, H-3), 4.20–4.38 (*m*, 2 H, H-6), 4.49 (*br s*, 1 H, H-5), 5.43 (*d, J* = 6.8 Hz, 1 H, H-4), 7.3-7.8 (*br s*, 2 H, OH). ^{13}C NMR ($CDCl_3$) δ 177.9, 173.3, 82.5, 72.2, 65.7, 42.9, 34.1, 31.4, 31.2, 29.2, 27.3, 24.6, 24.3, 22.5, 22.2, 13.9, 13.7. HRMS calcd. for $C_{17}H_{31}O_8P$, 394.3979; found 394.3970. *Anal.* calcd. for $C_{17}H_{31}O_8P$: C, 51.77; H, 7.92; P, 7.85. Found: C, 51.91; H, 7.99; P, 7.97.

Phosphoric acid, (3α,4β,5β)dihydro-5-[methyleneoxy-(hexanoyl)]-3-hexyl-2(3H)-furanone-4-(oxy) ester (**38**). Colorless oil (0.95 g, 90% yield). R_f = 0.76 (Solvent B). IR (neat, cm^{-1}) 3640–3000 (*br*), 2930, 2860, 1775, 1736, 1445, 1350, 1280, 1185, 1155, 990. ^1H NMR ($CDCl_3$) δ 0.80–0.95 (*m*, 6 H, 2 CH_3), 1.20–1.90 (*m*, 16 H, 8 CH_2), 2.37–2.37 (*m*, 2 H, CH_2), 2.75–2.90 (*m*, 1 H, H-3), 3.84–4.10 (*m*, 1 H, H-4), 4.30–4.46 (*m*, 2 H, H-6), 4.62–4.92 (*m*, 1 H, H-5), 7.2–7.8 (*br s*, 2 H, OH). ^{13}C NMR ($CDCl_3$) δ 176.4, 174.4, 79.6, 76.2, 62.6, 47.8, 34.1, 31.4, 31.2, 28.9, 28.3, 26.6, 24.4, 22.5, 22.2, 14.0, 13.8. HRMS calcd. for $C_{17}H_{31}O_8P$, 394.3979; found 394.3968. *Anal.* calcd. for $C_{17}H_{31}O_8P$: C, 51.77; H, 7.92; P, 7.85. Found: C, 51.88; H, 7.74; P, 7.81.

Phosphoric acid, (3α,4β,5β)dihydro-4-[oxy(hexanoyl)]-3-hexyl-2(3H)-furanone-5-(methyleneoxy) ester (**39**). Colorless oil (0.44 g, 99% yield). R_f = 0.69 (Solvent B). IR (neat, cm^{-1}) 3620–3000 (*br*), 2920, 2845, 1772, 1725, 1460, 1355, 1160, 1030, 970. ^1H NMR ($CDCl_3$) δ 0.84–0.98 (*m*, 6 H, 2 CH_3). 1.20–1.52 (*m*, 12 H, 6 CH_2), 1.56–1.83 (*m*, 4 H, 2 CH_2), 2.37 (*dd, J* = 7.0, 7.4 Hz, 2 H, CH_2), 2.68–2.76 (*m*, 1 H, H-3), 4.18–4.30 (*m*, 2 H, H-6), 4.84–4.91 (*m*, 1 H, H-5), 5.30 (*dd, J* = 5.2, 4.9 Hz, 1 H, H-4), 7.90–8.40 (*br s*, 2 H, OH). ^{13}C NMR ($CDCl_3$) δ 178.5, 174.8, 89.3, 75.1, 65.7, 47.9, 35.1, 32.8, 32.5, 30.3, 29.9, 27.9, 25.7, 23.8, 23.7, 15.3, 15.1. HRMS calcd. for $C_{17}H_{31}O_8P$, 394.3979; found 394.3993. *Anal.* calcd. for $C_{17}H_{31}O_8P$: C, 51.77; H, 7.92; P, 7.85. Found: C, 51.84; H, 7.81; P, 7.93.

General procedure for the preparation of lactone phospholipids. Phosphoric acid, (3α,4α,5β)dihydro-4-oxyhexanoyl-3-hexyl-2(3H)-furanon-5-[methyleneoxy-2″-(trimethylamino)ethyl] ester (**43**). To a solution of the phosphoric acid lactone ester **40** (0.72 g, 1.83 mmol) in dry pyridine (32 mL), 2,4,6-triisopropylbenzenesulfonyl chloride (1.66 g, 5.48 mmol, freshly recrystallized from pentane containing 1% thionyl chloride) was added which was followed by choline tetraphenylborate (1.67 g, 3.66 mmol). The mixture was stirred at 35°C for 4 h with the flask tightly stoppered. Water (1 mL) was added, and the solution was concentrated *in vacuo* (18). The residue was purified twice on silica gel (Solvent B), the product containing fractions were concentrated, and the residue was lyophilized from C_6H_6 to give **43** (0.34 g, 43% yield) as a pale yellowish solid in the purity of 98.5% as judged by HPLC: R_f = 0.36 (Solvent B), m.p. 160–162°C. IR (KBr, cm^{-1}) 3640–3100 (*br*), 2980, 2845, 1772, 1725, 1460, 1355, 1240, 1165, 1050. ^1H NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 0.81–0.88 (*m*, 6 H, 2 CH_3), 1.18–1.88 (*m*, 16 H, 8 CH_2), 2.29 (*dd, J* = 7.5, 7.6 Hz, 2 H, CH_2), 2.90–3.0 (*m*, 1 H, H-3), 3.35 [*s*, 9 H, 2″-$N(CH_3)_3$], 3.78–3.86 (*m*, 2 H, 1″-CH_2), 4.23–4.30 (*m*, 2 H, 6-CH_2), 4.0–4.23 (*m*, 2 H, 2″-CH_2), 4.62–4.85 (*m*, 1 H, H-5), 5.37 (*d, J* = 6.9 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 177.7, 172.9, 83.3, 72.3, 66.9, 64.7, 59.3, 54.3 (3 C), 43.0, 34.2, 31.5, 31.2, 29.4, 27.5, 24.5, 24.3, 22.5, 22.2, 14.0, 13.7. HRMS calcd. for $C_{22}H_{42}O_8NP$, 479.2612; found 479.2638. *Anal.* calcd. for $C_{22}H_{42}O_8NP$: C, 55.14; H, 8.83; N, 2.92; P, 6.46. Found: C, 55.26; H, 8.91; N, 2.81; P, 6.55.

Phosphoric acid, (3α,4β,5β)dihydro-5-[methyleneoxy-(hexanoyl)]-3-hexyl-2(3H)-furanone-4-[oxy-2′-(trimethylamino)ethyl] ester (**41**). Light yellowish semi-solid (0.35 g, 36% yield) 98.2% pure by HPLC. R_f = 0.45 (Solvent B). IR (KBr, cm^{-1}) 3600–3200 (*br*), 2940, 2260, 1770, 1730,

1470, 1370, 1240, 1170, 1085. ^{1}H NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 0.84–0.95 (*m*, 6 H, 2 CH_3), 1.16–1.38 (*m*, 13 H, CH_2), 1.40–1.72 (*m*, 3 H, CH_2), 2.30 (*dd*, *J* = 7.5, 7.6 Hz, 2 H, CH_2), 2.76 (*ddd*, *J* = 2.1, 7.1, 7.2 Hz, 1 H, H_3), 3.38 [*s*, 9 H, 2′-$N(CH_3)_3$], 3.76–4.20 (*m*, 2 H, 1′-CH_2), 4.21–4.48 (*m*, 2 H, 6-CH_2), 4.32–4.40 (*m*, 2 H, 2′-CH_2), 4.68–4.80 (*m*, 2 H). ^{13}C NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 177.2, 173.7, 79.3, 76.2, 66.2, 63.2, 59.4, 54.3 (3 C), 48.7, 34.1, 31.4, 31.2, 29.1, 28.4, 26.9, 24.5, 22.5, 22.2, 14.0, 13.8. HRMS calcd. for $C_{22}H_{42}O_8NP$, 479.2612; found 479.2627. *Anal.* calcd. for $C_{22}H_{42}O_8NP$: C, 55.14; H, 8.83; N, 2.92; P, 6.46. Found: C, 55.21; H, 8.93; N, 2.86; P, 6.50.

Phosphoric acid. (3α,4β,5β)dihydro-4-[oxy(hexanoyl)]-3-hexyl-2(3H)-furanone-5-[methyleneoxy-2″-(trimethylamino)ethyl] ester (**42**). Light yellowish semi-solid (0.33 g, 68% yield) 99.0% pure by HPLC. R_f = 0.26 (Solvent B), m.p. 144–146°C. IR (KBr, cm^{-1}) 3660–3100 (*br*), 2960, 2860, 1775, 1730, 1480, 1380, 1250, 1170, 1090. ^{1}H NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 0.80–0.93 (*m*, 6 H, 2 CH_3), 1.18–1.35 (*m*, 10 H, 5 CH_2), 1.37–1.44 (*m*, 2 H, CH_2), 1.50–1.88 (*m*, 4 H, 2 CH_2), 2.30 (*ddd*, *J* = 1.7, 6.9, 7.6 Hz, 2 H, CH_2), 2.62 (*ddd*, *J* = 4.0, 6.1, 8.2 Hz, 1 H, H-3), 3.32 [*s*, 9 H, 2″-$N(CH_3)_3$], 3.72–4.10 (*m*, 2 H, 1″-CH_2), 4.20–4.30 (*m*, 2 H, 6-CH_2), 4.10 (*m*, 2 H, 2″-CH_2), 4.83–4.89 (*m*, 1 H, H-5), 5.22 (*dd*, *J* = 4.0, 5.3 Hz, 1 H, H-4). ^{13}C NMR ($CDCl_3/CD_3OD$, 3:1, vol/vol) δ 176.6, 172.9, 79.0, 74.0, 66.2, 63.5, 59.3, 54.3 (3 C), 46.8, 33.8, 31.4, 31.1, 28.9, 28.4, 26.7, 24.3, 22.5, 22.2, 13.9, 13.8. HRMS calcd. for $C_{22}H_{42}O_8NP$, 479.2612; found 479.2636. *Anal.* calcd. for $C_{22}H_{42}O_8NP$: C, 55.14; H, 8.83; N, 2.92; P, 6.46. Found: C, 55.28; H, 8.89; N, 2.98; P, 6.59.

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ARTICLE

Effect of Nonionic Detergents on Lipoxygenase Catalysis

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In many studies on lipoxygenase catalysis, nonionic detergents are used to obtain an optically transparent solution of the fatty acid substrate. In order to resolve some controversies that exist with regard to the interpretation of kinetic data obtained with solutions containing nonionic detergents, a systematic investigation was undertaken into the effects of Lubrol, Tween-20 and Triton X-100 (0–0.8 g/L) on the kinetics of linoleate (2.5–110 μM) dioxygenation, catalyzed by lipoxygenase-1 or lipoxygenase-2 from soybean, at pH 9 or 10, at 25°C. Under most conditions, it was found that the detergents slowed down the reaction. However, at high linoleate concentrations, where substrate inhibition of lipoxygenase is significant, small amounts of detergent increased the dioxygenation rate. In a quantitative analysis of the results, a kinetic model in which the incorporation of linoleate in the detergent micelles is formulated as a simple reversible equilibrium, and in which both lipoxygenase-1 and -2 interact with free linoleate, but not with linoleate incorporated in the micelles, appeared to be sufficient to predict experimental results over a wide range of experimental conditions. According to this model, the changes in the dioxygenation kinetics caused by the presence of nonionic detergents are similar (but not equal) to those caused by competitive inhibitors. The conclusions that monomeric, nonmicellar linoleate is the preferred substrate for lipoxygenase and that the observed inhibition and stimulation are solely due to changes in the effective linoleate concentration strongly corroborate the earlier observations by Galpin and Allen [*Biochim. Biophys. Acta* 488 (1977), 392–401].

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Lipoxygenases (EC 1.13.11.12) catalyze the dioxygenation of polyunsaturated fatty acids that contain one or more (1*Z*,4*Z*)-pentadiene systems. The reaction products are hydroperoxides with a conjugated (*E*,*Z*)-diene moiety (for reviews see Refs. 1–3). Soybean lipoxygenase-1, the most thoroughly characterized of all plant lipoxygenases, shows optimum activity at pH 9–11. At these pH values, the critical micelle concentration (CMC) of linoleic acid is high (50 μM at pH 9, 200 μM at pH 10; Ref. 4). However, many other lipoxygenases have a pH optimum around 7. At pH 7, the CMC of linoleic acid is much lower (<20 μM, Ref. 4), and the solutions are often turbid. In studies on lipoxygenases at neutral pH, nonionic detergents, such as Tween-20 or Triton X-100 (source information in Experimental Procedures section), are frequently added to the reaction mixture to obtain an optically transparent solution.

Detergents are known to affect the dioxygenation rate. Many investigators have observed inhibition and/or stimulation of the dioxygenation reaction by surfactants (e.g., Refs. 5–8), but controversy exists about the interpretation of the data. In two recent studies on the effect of surfactants on the lipoxygenase reaction (7,8), the observed inhibition and activation were attributed to direct interactions between the detergents and lipoxygenase. In their analysis of the data, the authors did not take into account the fact that linoleate is itself an amphiphile and can, therefore, interact with the detergent micelles. On the other hand, in 1977 Galpin and Allen (9) published a study on the influence of micelle formation on lipoxygenase kinetics in which they used *n*-alcohols and *n*-carboxylic acids to modify the CMC of linoleate. Their results provided convincing evidence that lipoxygenase interacts mainly with monomeric linoleate, and that virtually all of the inhibition or activation effects of the alcohols and carboxylic acids could be attributed to changes in the concentration of nonmicellar linoleate. Furthermore, in a recent analysis of lipoxygenase catalysis in reversed micelles, Perez-Gilabert *et al.* (10) found that their data were best predicted by a model in which linoleate dissolved in the water phase was the sole substrate for lipoxygenase-1, and interactions between lipoxygenase and interfacial linoleate were neglected.

In order to resolve the controversies concerning the interpretation of dioxygenation data collected in the presence of a detergent, we have studied the dioxygenation kinetics over a wide range of linoleic acid and detergent concentrations. In most experiments we have used the nonionic detergent Lubrol (source information in Experimental Procedures section), which does not absorb at the wavelength used to monitor the dioxygenation (243 nm), but we have also studied the effect of two other commonly used detergents, Tween-20 and Triton X-100. Furthermore, we recorded the pH profile for lipoxygenase-1 activity in buffers containing detergent and compared it with the profile obtained in the absence of detergent.

We present a quantitative analysis of the data obtained at pH 9 and 10. Because the Michaelis–Menten formulation, which is often used to describe dioxygenation data, is only valid under certain conditions (11), we have analyzed the data in terms of the more comprehensive two-step model for lipoxygenase catalysis (11–13).

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Abbreviations: CMC, critical micelle concentration; CMC_d, concentration of nonmicellar detergent; HPOD, hydroperoxyoctadecadienoic acid; SDS, sodium dodecylsulfate.

EXPERIMENTAL PROCEDURES

Materials. Lipoxygenase-1 was prepared from soybeans (White Hilum; Central Soya, Utrecht, The Netherlands) as described previously (Ref. 11 and references therein). The preparation had a specific activity of 200 μmol · min^{-1} · mg^{-1} at pH 9, and of 40 μmol · min^{-1} · mg^{-1} at pH 7 (measured at 100 mM linoleate). It showed one major band after sodium dodecylsulfate (SDS) gel electrophoresis upon Coomassie Brilliant Blue staining. Lipoxygenase-1 was stored in 0.1 M sodium acetate, pH 5.5, containing 134 g/L ammonium sulfate. Partially purified lipoxygenase-2 was obtained in the second ion exchange chromatographic step (DEAE-Sepharose; Pharmacia, Uppsala, Sweden) in the same purification procedure. Lipoxygenase-2 activity was found in a small peak preceding the lipoxygenase-1 peak. The protein in the lipoxygenase-2 peak was precipitated with 320 g/L ammonium sulfate, then resuspended in 0.1 M sodium, acetate pH 5.5, and used without further purification. The specific activity of the lipoxygenase-2 preparation was 1 mmol · min^{-1} · mg^{-1} at pH 6 (measured at the linoleic acid concentration at which the rate is maximal, 25 mM, see Fig. 3a later in article) and was 0.3 mmol · min^{-1} · mg^{-1} at pH 9.

Linoleic acid [(9*Z*,12*Z*)-octadecadienoic acid] was obtained from Janssen Chimica (Beerse, Belgium) and was stored under argon as a 300 mM solution in methanol p.a. (Merck, Darmstadt, Germany) at 4°C. Lubrol (unspecified polyoxyethyleneglycols from fatty alcohols) was purchased from ICN (Cleveland, Ohio), Tween-20 from Fluka (Buchs, Switzerland), and Triton X-100 from Serva (Heidelberg, Germany).

Kinetic measurements. A Hi-Tech (Salisbury, United Kingdom) SF-51 stopped-flow spectrophotometer was used for the kinetic experiments. The formation of hydroperoxylinoleate (HPOD) was followed by recording the absorbance at 243 nm (UG-5 filter, 45% transmission at 243 nm) in a 10-mm light-path observation chamber. The molar absorbance for HPOD at 243 nm was 17.8×10^3 M^{-1} · cm^{-1}. The absorbances at 243 nm of 1 g/L Tween-20 and 1 g/L Triton X-100 were 0.15 and 0.48 cm^{-1}, respectively. Measurements were performed at 25°C in 0.09 M Na-borate (pH 7.9–10.7) or 0.09 M sodium phosphate (pH 6.7–9.0) buffers. A small amount of methanol from the linoleic acid stock solution was carried through into the final reaction mixture (maximally 0.03% vol/vol). As methanol is known to have a slight effect on the kinetics of the lipoxygenase reaction (11), the methanol concentration was kept constant (0.03% vol/vol) in all reaction mixtures by adding an appropriate amount of extra methanol. The reactions were initiated by mixing lipoxygenase (in syringe 1) with linoleate and detergent (in syringe 2). The final lipoxygenase concentration was 50 nM, unless otherwise indicated.

Interpretation of the data. An analytical steady-state rate equation for lipoxygenase catalyzed oxygenation of linoleate was derived in previous studies (11,13). The steady-state rate, *r*, at given linoleate (*S*) and HPOD (*P*) concentrations is given by:

$$r = \frac{V_{max}[S]}{\{K_S^*(1 + [P]/K_P^*) + [S]\} + \alpha \cdot \{K_P(1 + [S]/K_S) + [P]\} \cdot [S]/[P]} \quad [1]$$

In this expression, V_{max} is the maximum rate, K_S^*, K_P^*, K_S and K_P are the dissociation constants of the iron(III)-lipoxygenase- (K^*) and iron(II)-lipoxygenase (K) -*S* and -*P* complexes. The parameter α is a constant, provided $[O_2]$ does not change by more than 20%.

All observed curves showed an initial increase in rate (the induction period), followed by a short period of maximum rate, r_{max} (note that r_{max} is not equal to V_{max}). Numerical simulations of the lipoxygenase reaction indicate that the steady-state approximation may be applied in analyzing r_{max} as a function of [linoleate] and [HPOD] (13). The observed values of r_{max} (indicated as *r* in the rest of the text) were fitted to the steady-state rate equation using a nonlinear least-squares program (Levenberg-Marquardt algorithm, Ref. 14) for evaluating functions of multiple independent variables. The values for [*S*] and [*P*] used in the fitting procedure were estimated from the absorbance at 243 nm at the start of the maximum rate period. Since the concentration of O_2 in water, at 25°C under air, is 240 μM, and *r* is measured at 3 μM < [*P*] < 5 μM, $[O_2]$ varies less than 5% in the experiments, and therefore the value of α is constant within the experimental error.

RESULTS

Effect of Lubrol on the dioxygenation rate at alkaline pH. The dioxygenation of 2.5–110 μM linoleate, catalyzed by 50 nM soybean lipoxygenase-1 at pH 10, was recorded in the absence of detergent. After completion of the reaction, all linoleate (within the experimental error of 5%) appeared to be converted into HPOD. The steady-state rates are shown in Figure 1a. The values of V_{max} and K_S^* were estimated from a nonlinear least-squares fit of these data to Equation 1 (Experimental Procedures section). Because the other parameters are highly correlated under the conditions of the experiments ($[O_2]$ = 240 μM and [HPOD] < 5 μM), they were entered as constants: K_P^* = 25 μM (11,13), K_P = 15 μM (15), K_S = 20 μM and α = 0.01 (11,13). The best fit values for V_{max} and K_S^* were 15.0 ± 0.2 μM/s and 20 ± 1 μM, respectively (Chi square = 1.5).

In Figure 1b we illustrate the effect of increasing concentrations of Lubrol (0.025–0.8 g/L) on the steady-state rate (r) at four different linoleate concentrations (9, 30, 55 and 110 μM). At all linoleate concentrations, the presence of detergent decreases the dioxygenation rate. However, all of the linoleate is eventually converted into HPOD. The effect of Lubrol at a concentration of 1.7 nM lipoxygenase is the same as the effect at 50 nM. The r_{max} values measured at 50 nM lipoxygenase were 30 times larger than the values measured at 1.7 nM lipoxygenase at all Lubrol concentrations.

The data presented in Figure 1 are not compatible with two simple inhibition modes in which the detergent acts directly on the enzyme (uncompetitive and simple noncompetitive inhibition). Competitive inhibition or a more

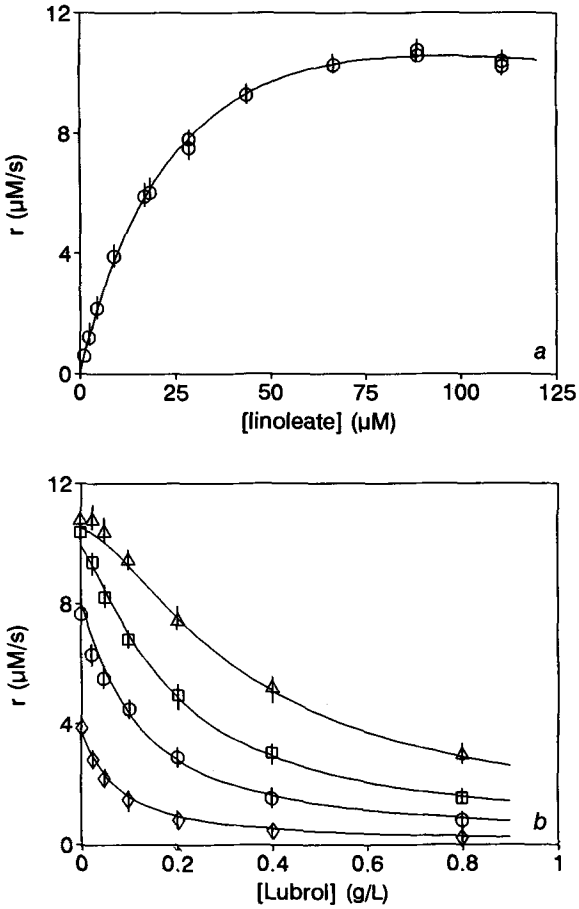

FIG. 1. Effect of Lubrol on the dioxygenation of linoleate catalyzed by 50 nM lipoxygenase-1. Reactions were carried out in 0.1 M sodium borate at pH 10. *a*: Steady-state rates in the absence of Lubrol at varying linoleate concentrations; circles, observed rates; solid lines show rates calculated with V_{max} = 15 μM/s, K_S^* = 20, K_P^* = 25, K_S = 20, K_P = 15 μM and α = 0.01. *b*: Steady-state rates measured at different Lubrol concentrations, starting with 9 (diamonds), 30 (circles), 55 (squares) and 110 (triangles) μM linoleate. Solid lines: rates calculated with the parameters mentioned under (*a*) and K_D = 32 μM, *f* = 770 μmol/g and CMC_d = 0. CMC_d, concentration of nonmicellar detergent.

complicated mode of enzyme inactivation is not excluded by the data. However, the presence of detergent would also slow down the reaction if micelle-bound linoleate were a poor substrate for lipoxygenase. Such a model would be in good agreement with the results of Galpin and Allen (9) and those of Perez-Gilabert *et al.* (10). In order to investigate this possibility, we used a kinetic model with the following features: (i) The fatty acid molecules interact with "fatty acid binding sites" on the detergent micelles in a simple reversible equilibrium, described by the dissociation constant K_D, (ii) only micelles can bind the fatty acid molecules, monomeric detergent molecules have no effect; (iii) the micelle-bound form of the fatty acid cannot interact with the enzyme; (iv) reactions between the fatty acid molecules and the detergent micelles are much faster than the enzyme-catalyzed reaction, and are not, therefore, rate limiting. For the sake of simplicity, we assume that the concentration of nonmicellar detergent (CMC_d) is constant at all linoleate concentrations. If 1 g of detergent can adsorb *f* μmoles of fatty acid, then B_0, the total concentration of "binding sites," is equal to $f \cdot (D - CMC_d)$, where *D* is the concentration of detergent in g/L. At a total fatty acid concentration A_0 μM, the concentration of free fatty acid [*A*] is given by:

$$[A] = \{[(K_D + B_0 - A_0)^2 + 4 \cdot K_D \cdot A_0]^{1/2} - (K_D + B_0 - A_0)\}/2 \quad [2]$$

In the nonlinear least-squares fit of the data with this model, the concentrations of free linoleate and HPOD ([*S*] and [*P*]) for use in Equation 1 were calculated from Equation 2 by substituting the appropriate total concentrations S_0 and P_0 for A_0. All data presented in Figure 1 were evaluated simultaneously (see Experimental Procedures section). The accuracy of the data does not allow separate estimations of K_D for Lubrol-linoleate and Lubrol-HPOD, because under the conditions of the measurements (3 < [HPOD] 5 μM), the rate is mainly determined by the concentration of linoleate. The best fit values are: V_{max} = 15.0 ± 0.2 μM/s, K_S^* = 20 ± 1 μM, *f* = 770 ± 80 μmoles/g and K_D = 32 ± 5 μM (Chi square = 2, but see below). The concentration of noninteracting detergent (CMC_d) must be very small, and cannot be determined accurately from these data. Its upper limit is estimated to be 10 mg/L. The experimental data satisfy the simple model very well. This can be seen in Figure 1b, in which the calculated data have also been indicated.

The values of K_D and *f* appeared to be correlated—other values of *f* and K_D also fit the data well, as long as f/K_D is approximately 25 L/g and K_D > 15 μM. Therefore, the absolute values of K_D and *f* (and their errors) have little physical significance. The correlation indicates that the total number of binding sites B_0 is much larger than the number of occupied sites, [*AD*], even at the highest concentration of linoleate used in these experiments. When $B_0 - [AD] \approx B_0$, the expression for the concentration of free linoleate becomes:

$$[A] = A_0/(1 + (D - CMC_d) \cdot f/K_D) \quad [3]$$

When Equation 3 was used instead of Equation 2 for the calculation of free linoleate, the fit of the data was equally good (Chi square = 2). The best fit value for f/K_D was 21 ± 5 L/g.

When the rate is measured as a function of the linoleate concentration and the concentration of detergent is constant, the observed rates coincide with the predicted values within the experimental error. However, at low substrate concentrations (<15 μM), the observed rates are systematically somewhat lower, and at high substrate concentrations (50–100 μM), the values are slightly higher than the predicted rates (not shown). This effect may be due to exclusion phenomena such as those described, for example, by McGhee and Von Hippel (16). Binding of one molecule of linoleate to a detergent micelle presumably excludes binding of other linoleate molecules to potential "binding sites" in its vicinity. In that case, f/K_D is a function of the linoleate

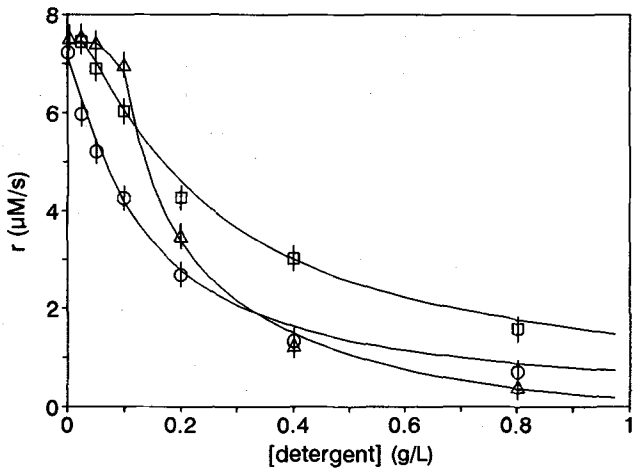

FIG. 2. Comparison of the effects of Lubrol (circles), Tween-20 (squares) and Triton X-100 (triangles) on the steady-state oxygenation rates. Reactions were performed with 50 nM lipoxygenase-1 and 30 μM linoleate in 0.1 M sodium borate, pH 10. Solid lines show rates calculated with parameters obtained from best fits of data obtained at 10, 30, 50 and 100 μM linoleate: f/K_D = 24 L/g, CMC_d = 0 (for Lubrol data); f/K_D = 10 L/g, CMC_d = 0.031 g/L (for Tween-20 data) and f/K_D = 30 L/g and CMC_d = 0.091 g/L (for Triton X-100 data). All other parameters and abbreviation are as in Figure 1.

concentration rather than a constant, and the estimated value for f/K_D is merely an average.

Effect of other nonionic detergents. Because Triton X-100 and Tween-20 are also commonly used to solubilize linoleic acid, we investigated whether the model described for the action of Lubrol holds for these compounds. We measured the dioxygenation rates at 10, 30, 50 and 100 μM linoleate, in the presence of 0–0.8 g/L detergent. In Figure 2 the effects of increasing concentrations of Tween-20 or Triton X-100 are compared with the results obtained for Lubrol (see Fig. 1b, linoleate concentration, 30 μM). Addition of less than approximately 0.05 g/L Tween-20 or 0.1 g/L Triton X-100 has little effect (all linoleate concentrations), but higher concentrations of either detergent cause the dioxygenation rate to decrease. After completion of the reactions, all of the linoleate (within the experimental error of approximately 5%) had again been converted into HPOD. The data for Tween-20 and for Triton X-100 (all linoleate concentrations) were successfully fitted to Equations 1 and 2. The best fit values for K_D and f were correlated, and the use of Equation 3 instead of Equation 2 gave equally good results. The best fit values of f/K_D are 10 L/g for the Tween-20 data, and 30 L/g for the Triton X-100 data. The best fit values for CMC_d were 0.031 ± 0.008 and 0.091 ± 0.003 g/L, respectively. These values are lower than the CMCs that have been reported for these detergents in pure form (0.06 g/L for Tween-20 and 0.19 g/L for Triton X-100, Refs. 17 and 18). However, the concentration of monomeric detergent in equilibrium with mixed micelles is always lower than the CMC of the pure detergent (19). Furthermore, we used commercial detergent preparations, whose CMCs may have been smaller than the published values, owing to inhomogeneity or impurities.

Effect of Lubrol on the lipoxygenase-2 reaction. If the inhibition of the dioxygenation reaction by Lubrol is solely due to adsorption of the fatty acid substrate, then the effect of Lubrol on the reaction catalyzed by other lipoxygenases must be predictable from the data obtained with lipoxygenase-1. Therefore, we investigated

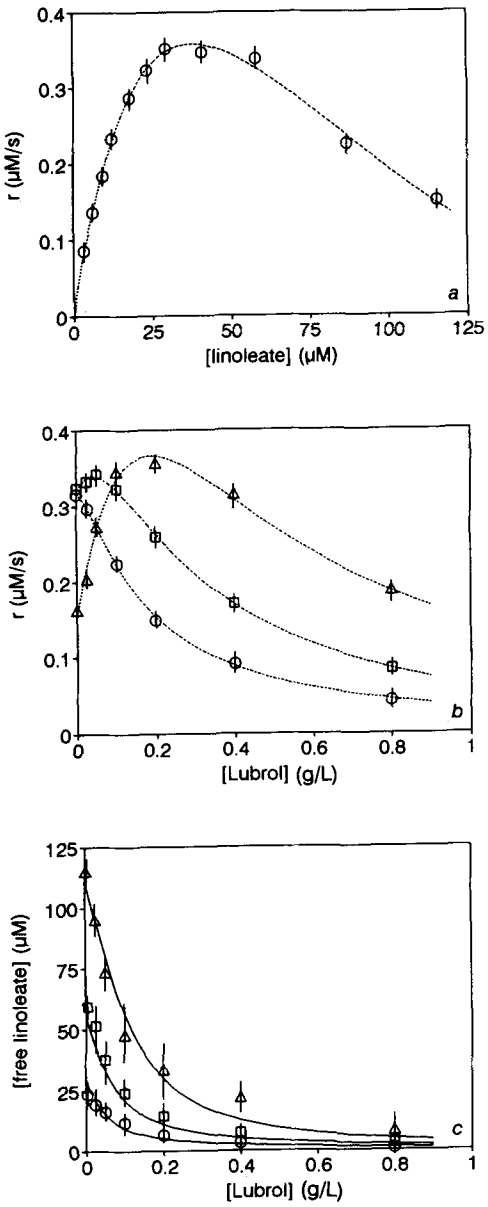

FIG. 3. Effect of Lubrol on the dioxygenation of linoleate, catalyzed by lipoxygenase-2. Data obtained with a partially purified enzyme preparation in 0.1 M sodium borate at pH 9. *a*: Rates in the absence of Lubrol at varying linoleate concentrations. *b*: Rates measured in the presence of varying Lubrol concentrations, starting with 30 (circles), 55 (squares) and 110 (triangles) μM linoleate. *c*: Comparison of concentrations of free (nonmicellar) linoleate, estimated from the data obtained with lipoxygenase-2 with calculated values. Solid lines: calculated concentrations (K_D = 32 μM and f = 770 μmol/g). Other symbols: concentration of free linoleate estimated by comparing the rates from *b*, those obtained in the absence of Lubrol (*a*). Total linoleate concentrations: 30 (circles), 55 (squares) and 110 (triangles) μM.

the effect of Lubrol on the reaction catalyzed by lipoxygenase-2 from soybean.

Lipoxygenase-1 and -2 have different substrate specificities (20) and form different products (21). In the lipoxygenase-2 reaction, 10–35% of all linoleate is converted into compounds other than HPOD (see Ref. 22), as judged by the increase of absorbance at 285 nm during the reaction (in the lipoxygenase-1 reaction, over 95% of the linoleate is converted into HPOD). The steady-state kinetics are also different. A plot of the steady-state dioxygenation rate for lipoxygenase-2 as a function of linoleate concentration, obtained at pH 9 and at a protein concentration of 0.25 μg/mL, is shown in Figure 3a. The curve shows a marked decrease in rate at linoleate concentrations greater than 25 μM. A similar decrease in rate appears in the lipoxygenase-1 reaction, but only at linoleate concentrations above 100 μM (not shown). The rate decrease is due to substrate inhibition (11). This effect is much more pronounced for lipoxygenase-2 reaction than for lipoxygenase-1.

Addition of Lubrol to the lipoxygenase-2 reaction mixture merely slows down the reaction when the linoleate concentration is 30 μM (Fig. 3b). However, at a linoleate concentration of 55 μM, a slight stimulation of the reaction is observed at Lubrol concentrations up to 0.05 g/L. Concentrations greater than 0.1 g/L are again inhibitory. A much larger stimulation is observed in the reaction with 110 μM linoleate. At a Lubrol concentration of 0.2 g/L, the rate is twice as high as in the absence of detergent. At higher Lubrol concentrations, the reaction proceeds more slowly than at 0.2 g/L Lubrol. In spite of the apparently different effects of Lubrol on lipoxygenase-1 and -2, the results can still be explained with the same model. The micelles adsorb a certain fraction of the linoleate, which cannot then be bound by lipoxygenase. When the total linoleate concentration is smaller than 25 μM, addition of Lubrol only results in a reduced reaction rate. At higher linoleate concentrations, substrate inhibition of lipoxygenase-2 is substantial. Decreasing the concentration of free linoleate will result in a decreased substrate inhibition and, thus, in a rate increase. Addition of more detergent eventually results in a rate decrease, because the concentration of free linoleate drops below 25 μM.

Because of the lack of sufficient *a priori* knowledge of the values of K_P^*, K_S, K_P and α for the lipoxygenase-2 reaction, it is not possible to perform a quantitative analysis similar to the one applied to the lipoxygenase-1 data. Assuming that the model for the action of detergents is correct, it is possible to estimate the concentrations of free linoleate simply by comparing the rates observed in the presence of Lubrol with the values obtained in the absence of detergent (Fig. 3a). The dioxygenation rate in the absence of detergent at 35–60 μM linoleate is approximately constant (0.35 ± 0.2 μM/s). Therefore, the estimate of the concentration of free linoleate corresponding to a rate of 0.35 ± 0.2 μM/s is rather inaccurate (48 ± 13 μM). The error on the other estimates is smaller (approximately 5 μM for estimates smaller than 25 μM, 10 μM for estimates above 70 μM). In Figure 3c we compare these estimates with values calculated from Equation 2, with K_D = 32 μM and f = 770 μmol/g, the parameter values calculated for the lipoxygenase-1 reaction. The concentrations of free linoleate estimated from the dioxygenation rates agree very well with the ones calculated with Equation 2. This indicates that the fraction of linoleate incorporated in micelles is independent of the type of lipoxygenase used in the assay.

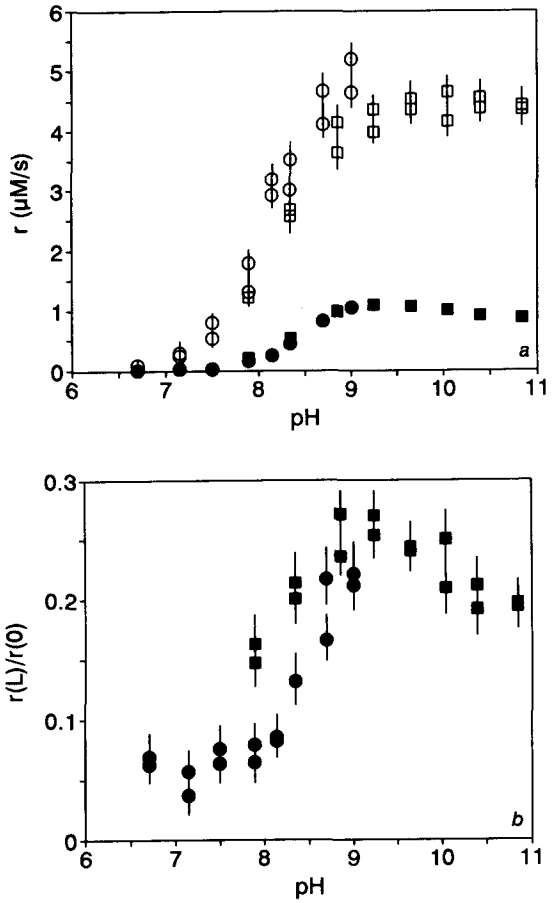

FIG. 4. Effect of pH. *a*: Steady-state dioxygenation rates in the absence or presence of 0.2 g/L Lubrol. The concentration of lipoxygenase-1 was 50 nM, the total linoleate concentration was 10 μM. Open symbols, no Lubrol; closed symbols, 0.2 g/L Lubrol; circles, 0.1 M Na-phosphate buffers; squares, 0.1 M Na-borate buffers. *b*: Ratio r(L)/r(0) of rates in buffers with [r(L)] and without [r(0)] 0.2 g/L Lubrol.

The effect of pH. The effect of detergent on the pH profiles of lipoxygenase-1 is shown in Figure 4. The profile was obtained at 10 μM linoleic acid. We used this low-linoleate concentration to minimize effects due to substrate inhibition or aggregation. The plots show a steep increase in rate between pH 7 and 9. The rates measured in the presence of Lubrol are significantly lower (Fig. 4a). If the composition of the detergent-fatty acid micelles were independent of pH, the concentration of free fatty acid would be the same at each pH. In that case, the ratio of the rates in buffers with or without Lubrol would be constant. However, this ratio varies quite strongly (Fig. 4b). At pH <7.5, dioxygenation in the absence of Lubrol is approximately 20 times faster than in the presence of Lubrol. At pH >9, the rate in the absence

of Lubrol is only 4 to 5 times higher. This means that the concentration of free lipid substrate is significantly smaller at pH <7.5 than at pH >9.

It has been proposed (6) that the activity of soybean lipoxygenase-1 depends on the charge of the fatty acid substrate. Lipoxygenase-1 reacts best with charged substrates (such as the linoleate anion), but seems to have a much lower affinity for uncharged compounds (such as unionized linoleic acid). The apparent pK_a value for linoleic acid is 7.9 (6). The pH profile of lipoxygenase-1 obtained in the absence of detergent is consistent with this. One would also expect that the amount of linoleic acid incorporated in the mixed micelles would decrease upon increasing the pH, owing to the repulsive interactions between the negatively charged head groups. The curve of the ratios (Fig. 4b) has the same sigmoidal shape as the activity profile (Fig. 4a), because the presence of detergent merely exaggerates the effect of pH on the lipoxygenase-1 activity. However, the presence of Lubrol can cause a shift in the pH optimum of lipoxygenases that have highest affinity for less polar substrates (unpublished observations). The results presented here do not exclude the possibility that Lubrol has some pH-dependent effect on lipoxygenase itself, nor that the pK_a of linoleic acid is affected by the presence of Lubrol. Nonetheless, the experiments indicate that pH profiles obtained in buffers containing detergent are different from those obtained in buffers without detergent.

DISCUSSION

The results presented above strongly favor a model in which lipoxygenase has a substantially higher affinity for monomeric substrate than for fatty acid incorporated in micelles. A quantitative model, in which the detergent simply decreases the effective substrate concentration, accurately predicts the results for more than one type of lipoxygenase, without requiring any further assumptions. It is not possible to reconcile the present results with an equally simple model in which a detergent acts directly on the enzyme. Such a model would require many assumptions, for which no experimental evidence exists, to explain the observed inhibition and stimulation.

The influence of micelle formation on lipoxygenase kinetics was studied by Galpin and Allen (9). Our findings confirm their conclusion that the effects of amphiphiles on the dioxygenation reaction could be ascribed to physicochemical interaction of these compounds with the substrate rather than to a direct interaction with the enzyme itself.

In many studies on lipoxygenase, a nonionic detergent is routinely added to solubilize the substrate. If quantitative conclusions are to be drawn from the experiments, it is essential to characterize the system in detail. When such a characterization is omitted, it is quite possible that phenomena caused by changes in the effective substrate concentration are incorrectly attributed to direct interactions between the enzyme and the detergent. For instance, the rate increase that is observed after addition of small amounts of detergent at high fatty acid concentrations (see Fig. 3) is easily mistaken for direct stimulation of the lipoxygenase activity (cf. Ref. 8). Furthermore, changes in K_m and V_{max} may be erroneously interpreted as indicative for competitive inhibition, for the following reason: The steady-state rate equation (Equation 1) for lipoxygenase-1 catalysis simplifies to the Michaelis-Menten equation for linoleate concentrations below 75 μM and HPOD concentrations from 3 to 5 μM, because the second term in the denominator and the value of $[P]/K_P^*$ are both small (11). Substitution of the substrate concentration in the Michaelis-Menten equation with the expression for $[A]$ from Equation 3 yields an equation that is identical to the expression for the rate in the presence of a competitive inhibitor. Therefore, the model predicts that, under certain conditions, the presence of detergents causes changes in K_m and V_{max} that are very similar to those caused by competitive inhibitors. As the Michaelis-Menten equation is only an approximation, straight lines generated in a Lineweaver-Burk plot will intersect slightly off the ordinate (which is generally interpreted as "mixed inhibition"; cf. Ref. 8). However, the results obtained with lipoxygenase-2 exclude competitive inhibition. A competitive inhibitor would enhance the effect of product and substrate inhibition, and merely decrease the dioxygenation rate.

In the interpretation of results of experiments in which detergents are used, it should also be taken into account that: (i) pH profiles obtained in buffers containing detergent are different from those obtained in buffers without detergent; (ii) lipoxygenase inhibitors and activators may associate with micelles and therefore seem to be less effective than in the absence of detergent; and (iii) it is possible that the addition of negatively charged amphiphiles to the nonionic detergent/linoleate solution will limit the amount of fatty acid that can be incorporated into the micelles, and thus stimulate the reaction (23). Positively charged amphiphiles may have the opposite effect (8).

It has been reported that some mammalian lipoxygenases are able to oxygenate biological membranes without prior action of a phospholipase (24). From the present study, it seems highly likely that lipoxygenase-1 and -2 from soybean interact preferentially with free linoleate, and not with linoleate included in a matrix of (nonionic) amphiphiles. Therefore, investigations are underway into the ability of plant lipoxygenases to oxygenate artificial and biological lipid bilayers.

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Effect of n-3 Fatty Acid-Rich Fish Oil Supplementation on the Oxidation of Low Density Lipoproteins

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This study was aimed at determining the effect of fish oil supplementation on copper-catalyzed oxidation of low density lipoproteins (LDL) from nine hypertriglyceridemic human subjects. A rapid headspace gas chromatographic method was used to measure the volatile oxidation products from LDL. Propanal and hexanal were the major volatile products formed in the oxidation of n-3 and n-6 polyunsaturated fatty acids (PUFA), respectively. Fish oil supplementation resulted in a significant increase in propanal formation from 3.7 to 13.4 nmol/mL LDL ($P < 0.01$); it also resulted in small decreases in pentanal formation from 14.7 to 11.4 nmol/mL LDL and in hexanal formation from 138 to 108 nmol/mL LDL ($P < 0.05$). The changes in peroxidation products paralleled the changes in LDL composition, which showed a significant increase in n-3 PUFA from 3.2 to 14.6% ($P < 0.01$) and a decrease in n-6 PUFA from 43.7 to 35.0% ($P < 0.05$). Propanal formation was highly and significantly correlated with n-3 PUFA content ($r = 0.950$, $P < 0.001$). Since total volatiles remained unchanged, this indicated that the two groups of LDL samples did not differ in overall oxidative susceptibility. Although fish oil intake did not alter the oxidative susceptibility of LDL, the chemically modified LDL particles generated a distinct pattern of volatile oxidation products that reflected changes in their fatty acid composition.

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Modification of low density lipoproteins (LDL) by oxidation of their polyunsaturated lipid components has been implicated in the etiology of atherosclerosis (1–3). Oxidation proceeds by interaction of polyunsaturated fatty acids (PUFA) with active oxygen species or with lipid oxidation products. Moreover, there are indications that lipid oxidation promotes premature coronary atherosclerosis since susceptibility to LDL oxidation has been associated with the severity of coronary heart disease (4). Protection against oxidation can come from antioxidants, antioxidant enzymes and cofactors (5). The use of antioxidants to inhibit LDL oxidation has been reviewed extensively (2,6). Under *in vivo* conditions, there exists a competition between oxidative and protective processes that depend on PUFA composition and on antioxidant levels.

Oxidation of human LDL leads to the formation of various aldehydes and to fluorescent apoprotein B (apo B) (7). This fluorescence is assumed to come from the Schiff bases formed by reaction of aldehydes with the lysine residues of apo B. Several studies have demonstrated that the fatty acid composition of LDL is influenced by diets rich in oleic acid (8–10) and by supplementation with fish oil n-3 PUFA (11–13). Oleate-rich LDL particles from diets rich in oleic acid were reported to be highly resistant to oxidation based on formation of thiobarbituric acid-reactive substances (TBARS) (8–10) and conjugated dienes. The oleate-rich LDL particles were also resistant to oxidaton induced by endothelial cells (8,9). Oxidation of LDL from smokers fed fish oil resulted in significantly higher TBARS than from nonsmokers fed fish oil (11). In another study (12), the susceptibility to Cu^{2+}-catalyzed peroxidation as measured by TBARS was similar in LDL from subjects on fish oil or on corn oil diets.

Although the validity of TBARS as an index of lipid peroxidation in biological samples has been a matter of considerable debate (14), the TBARS assay has been used most commonly to measure the degree of oxidation and the susceptibility to oxidation of LDL. However, the lack of specifity of the thiobarbituric acid (TBA) test makes results difficult to interpret (5,15).

We previously described a rapid method using headspace gas chromatography (GC) for the determination of hexanal, an important decomposition product in n-6 PUFA peroxidation. We used this method to examine rat liver samples (16), human red blood cell membranes (17) and human LDL (18). In this latter study, LDL from ten individuals showed a wide variation in susceptibility to oxidation, and hexanal values correlated significantly with total PUFA, 18:2 and n-6 PUFA contents of LDL, but poorly with 20:4 and vitamin E levels (18). Thus, we concluded that dietary PUFA is an important factor in determining the oxidative susceptibility of human LDL. However, vitamin E is not the sole determinant of oxidative susceptibility, and other endogenous antioxidants or inhibitors may contribute to the resistance of LDL to oxidation (18). Consistent with this we have shown, for example, that phenolic compounds in red wine contribute antioxidant activity and inhibit *in vitro* oxidation of human LDL (19).

With the new methodology on hand, we can now address the question whether LDL composition does affect the oxidative stability of lipoprotein particles. The present paper reports the application of headspace GC in a nutritional study to test the effect of fish oil supplementation on the susceptibility of LDL fractions to oxidation. Appearance of specific volatile peroxidation products was found to be correlated with the fatty acid profile but not with the tocopherol content of LDL.

MATERIALS AND METHODS

Blood was obtained from hypertriglyceridemic subjects who were enrolled in a 12-week, double blind, randomized

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Abbreviations: Apo B, apoprotein B; GC, gas chromatography; LDL, low density lipoproteins; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; UV, ultraviolet.

clinical trial (University of California at Davis Human Subjects Committee No. 92-086). Blood samples were taken during a period after the subjects had consumed 5.1 g of fish oil each day for 6 wk (supplemented). Unsupplemented samples were taken when fish oil was absent from the diet. The fish oil supplement contained 58.9% PUFA, including 8.6% n-6 PUFA and 44.3% n-3 PUFA (comprising 28.2% 20:5n-3 and 11.6% 22:6n-3), and 25 IU vitamin E. Plasma was separated from blood, and LDL fractions were prepared by density ultracentrifugation as described previously (18).

Prior to oxidation, LDL was exhaustively dialyzed with deoxygenated phosphate-buffered (10 mM, pH 7.4) saline (100 mM) for 24 h. The final concentration of each LDL sample was diluted with phosphate-buffered saline (10 mM) to the same protein concentration (1 mg/mL LDL). The freshly prepared LDL fractions from the unsupplemented and fish oil-supplemented periods were compared by the headspace GC method (18) to determine the volatile products formed by oxidation in the presence of copper. Samples of LDL in phosphate-buffered saline in the presence of 80 μM $CuSO_4$ were sealed in headspace bottles and incubated in a water bath shaker at 37°C. After incubation, the headspace bottles were pressurized with helium as carrier gas for 30 s, and an aliquot of the gas phase was injected into the gas chromatograph.

The fatty acid composition of LDL fractions was determined as described previously (18); vitamin E contents were measured by high-performance liquid chromatography (20). The least significant difference between mean values was determined by analysis of variance (21) of duplicate analyses.

RESULTS

To investigate the effect of LDL composition on the oxidative susceptibility of lipoprotein particles, a method is required that can discriminate between the various peroxidation products derived from different lipid components. The headspace GC technique was used to measure the volatile peroxidation products formed from n-6 PUFA and n-3 PUFA upon copper-catalyzed oxidation of human LDL. The products of n-6 PUFA have been shown to include pentane, pentanal and hexanal. The main product of n-3 PUFA oxidation is propanal (22). To compare the susceptibility to oxidation of human LDL samples, total volatiles were also determined by summation of the peak areas of all the volatiles detected.

A representative gas chromatographic analysis of the products of copper-oxidized LDL from a human subject on a fish oil-supplemented diet shows that the peak due to propanal increased dramatically (Fig. 1b) when compared to the LDL sample from the same subject on the unsupplemented diet (Fig. 1a). In both the supplemented and unsupplemented LDL samples, hexanal was the main volatile. This result is consistent with the fatty acid analyses showing an abundance of n-6 PUFA in the LDL samples (Table 1). Similarly, in the oxidized LDL from the subjects consuming the unsupplemented diet, the peaks due to pentane and pentanal were both larger than the peak due to propanal. Since pentane and pentanal reflect the

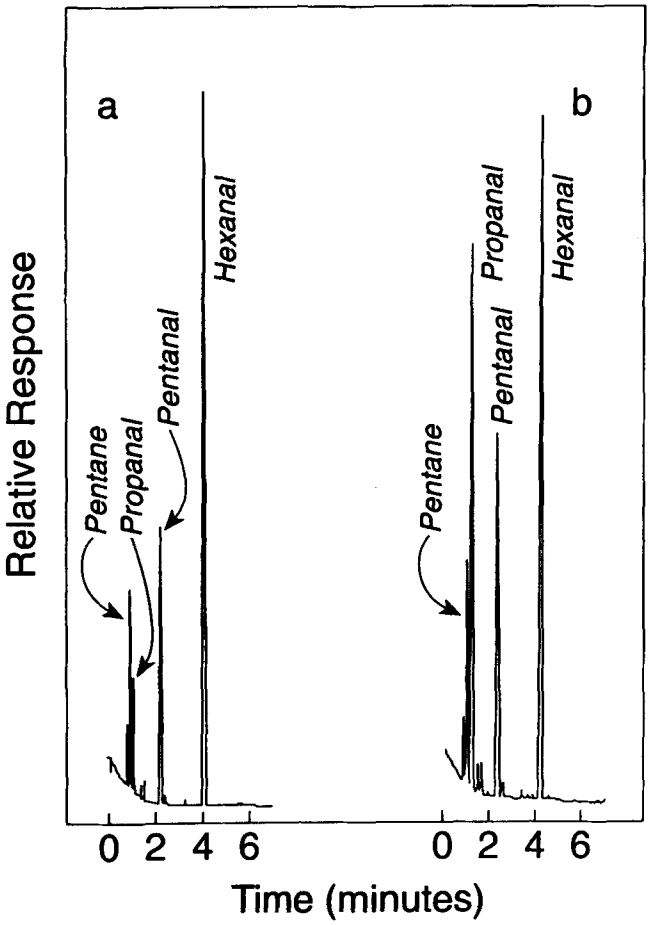

FIG. 1. Headspace gas chromatograms of human low density lipoprotein after incubation for 2 h at 37°C in the presence of 80 μM $CuSO_4$; a, unsupplemented subject; b, fish oil supplemented subject.

oxidation of n-6 PUFA while propanal reflects n-3 PUFA oxidation, headspace GC allowed us to determine the origin of various volatile products of lipid oxidation.

Figure 2 shows time plots of propanal and hexanal formation obtained from six samples of LDL oxidized with copper. After 2 and 4 h of oxidation at 37°C, propanal formation was more pronounced in LDL from subjects that had consumed a fish oil-supplemented diet than those that had consumed the unsupplemented diet (Fig. 2a). On the other hand, hexanal formation did not vary as much as propanal formation but after 2 and 4 h of oxidation it tended to be higher in the LDL samples from unsupplemented diets than in the LDL samples from fish oil-supplemented diets (Fig. 2b). The most consistent differences between the two types of LDL samples were observed after 2 h of oxidation.

Variation in oxidative susceptibility and the nature of the volatile peroxidation products were studied in nine LDL samples from subjects that had consumed an unsupplemented diet, and in nine samples from subjects that had consumed a fish oil-supplemented diet. The headspace GC analyses of the volatile products formed were compared with the fatty acid composition and the vitamin E contents of the lipid extracts (Table 1). A significant,

TABLE 1

Effect of Fish Oil Supplementation on LDL Oxidation

Analyses	Unsupplemented (n = 9)	Supplemented (n = 9)	$P <$
Volatiles (nmol/mL LDL)[a]			
Pentane	9.6 ± 4.2	7.5 ± 2.1	N.S.
Propanal	3.7 ± 1.1	13.4 ± 3.7	0.01
Pentanal	14.7 ± 6.8	11.4 ± 2.7	0.05
Hexanal	138 ± 41	108 ± 22	0.05
Total[b]	189 ± 51	189 ± 41	N.S.
Fatty acids (%)			
n-6 PUFA	43.7 ± 3.2	35.0 ± 3.5	0.05
n-3 PUFA	3.2 ± 0.6	14.6 ± 2.3	0.01
Total PUFA	46.8 ± 3.1	49.6 ± 2.9	N.S.
Tocopherol (nmol/mL LDL)	14.0 ± 5	18.0 ± 7	N.S.

[a]Contained 1 mg low density lipoprotein (LDL) protein/mL; determined after 2 h oxidation (see Figs. 1 and 2). N.S., not significant; PUFA, polyunsaturated fatty acids.

[b]Expressed as hexanal.

3.6-fold higher propanal production was observed in the LDL fractions from subjects after consumption of the fish oil supplement than from subjects on the unsupplemented diets ($P < 0.01$). This increase in propanal production coincided with a statistically significant 4.6-fold increase in n-3 PUFA content of LDL ($P < 0.01$). Small but significantly higher pentanal and hexanal levels ($P < 0.05$) were also observed in the oxidized LDL from the unsupplemented than the supplemented periods, consistent with the significantly higher content of n-6 PUFA in the LDL from subjects without fish oil supplementation. No significant changes were observed in pentane formation or total PUFA or tocopherol content between LDL samples from subjects on supplemented and unsupplemented diets.

The total volatiles were not different between the two groups of LDL samples. Thus, based on total volatiles generated, fish oil supplementation had no effect on the oxidative susceptibility of LDL from nine subjects with hypertriglyceridemia. Yet fish oil supplementation had a dramatic effect on the types of volatile oxidation products formed from LDL.

The correlation between propanal and n-3 PUFA was significant ($r = 0.950$, $P < 0.001$) (Fig. 3). Propanal values of nine oxidized LDL samples from subjects on unsupplemented diets varied only from 2.3 to 4.6%, while those from subjects on fish oil-supplemented diets varied from 11 to 17%. These large differences between LDL from subjects on unsupplemented diets and LDL from subjects on fish oil-supplemented diets are consistent with the significant increase in n-3 PUFA content of LDL (Table 1).

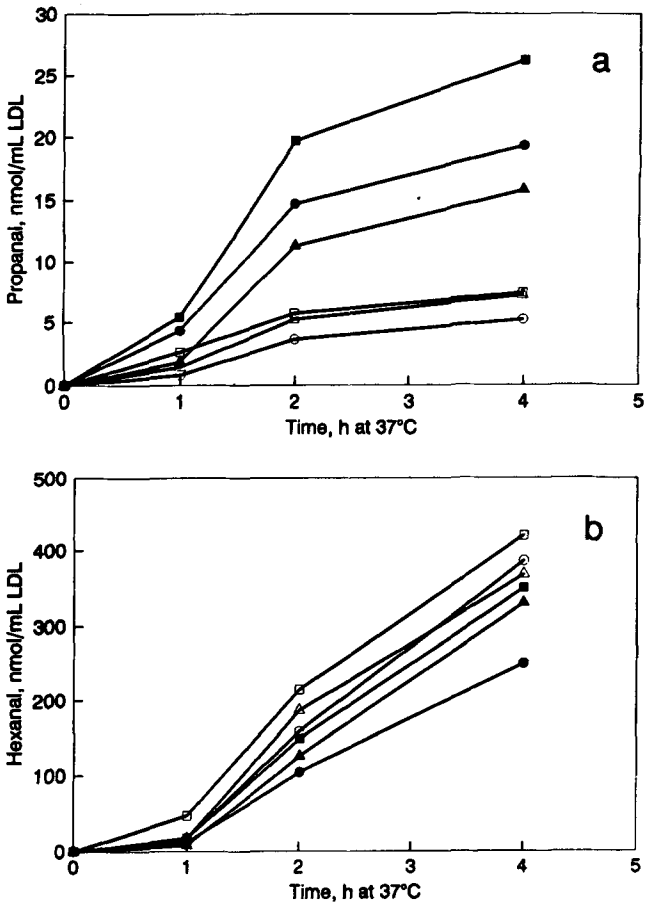

FIG. 2. Kinetics of volatile formation in six samples of human low density lipoprotein (LDL) oxidized in the presence of 80 μM $CuSO_4$; a, propanal; b, hexanal. Lines with closed symbols represent individual subjects consuming fish oil supplements, and lines with open symbols represent individual subjects on unsupplemented diets.

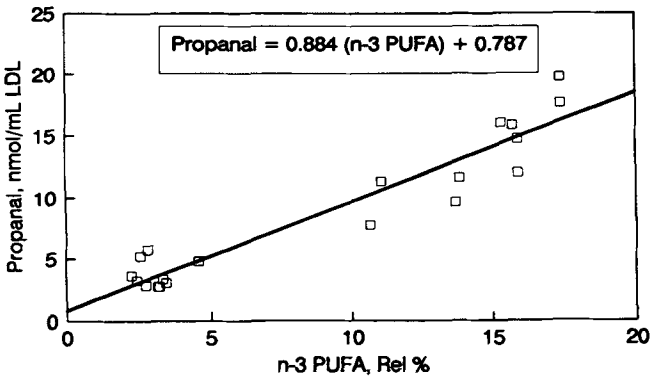

FIG. 3. Correlation of propanal vs. n-3 polyunsaturated fatty acids (PUFA) ($r = 0.950$, $P < 0.001$). LDL, low density lipoproteins.

DISCUSSION

In the present study, fish oil supplementation produced marked changes in the profiles of volatile peroxidation products of LDL from hypertriglyceridemic subjects. These changes in the distribution of oxidation products paralleled the altered fatty acid composition of LDL lipids. Thus, significant increases in propanal formation were observed during oxidation with copper in LDL from subjects supplemented with fish oil (Table 1). This increase in propanal can be directly attributed to increases in n-3 PUFA in the LDL lipids.

In spite of the marked changes in the types of aldehydes formed in the peroxidation of different LDL samples, no changes were observed in the formation of total volatiles. Therefore, there was no apparent difference in the overall oxidative susceptibility of LDL between subjects consuming fish oil and those on the unsupplemented diets. This result is consistent with the similar total PUFA levels observed in the two groups of LDL samples.

The headspace GC procedure used in the present work can be compared to the ultraviolet (UV) conjugated diene method that is used to measure the lag phase to assess the oxidative susceptibility of LDL and the effects of dietary antioxidants (3). In contrast to the UV method, the headspace GC procedure is based on the amounts of volatile oxidation products formed after the lag phase (Fig. 2), when the antioxidants are usually depleted. Thus, the headspace GC method may be expected to provide data that are more closely related to the variation in fatty acid composition of LDL than to the antioxidant protection.

Although the effect of n-3 PUFA from fish oils in reducing the plasma triglyceride level is well established (23–25), only a few reports have appeared on their effects on the oxidative susceptibility of human LDL. One study reported that LDL from human subjects consuming fish oil and corn oil showed similar susceptibility to copper-catalyzed oxidation based on lipid peroxide determinations (12). However, other studies reported significant increases in the formation of TBARS resulting from fish oil diets (11,13). Because TBARS are formed by oxidative degradation of PUFA containing more than two double bonds (26), the oxidation of any lipids containing n-3 PUFA would be expected to produce high levels of TBARS. Therefore, TBARS cannot be used as the sole determinant of oxidative susceptibility to compare the effects of dietary n-6 vs. n-3 PUFA on LDL oxidation. For the same reason the TBA assay cannot be used to estimate the oxidation of dietary oleic acid in LDL oxidation (8–10).

At the level of fish oil supplementation used in the prsent study, although n-3 PUFA from fish oils were incorporated into the LDL, no significant changes were observed in either LDL total PUFA or overall oxidative susceptibility based on total volatile production as determined by headspace GC. Thus, with adequate antioxidant protection, replacement of significant quantities of n-6 with n-3 PUFA may not represent a dramatic enhancement in oxidative susceptibility. However, whether or not the n-3 unsaturated aldehydes expected from oxidation of n-3 PUFA may be more reactive with proteins such as apo B than those produced from n-6 PUFA is not known. Treatment of fresh LDL with 2,4-heptadienal, a product of 18:3n-3 PUFA oxidation (22), led to a fluorogenic apo B, while hexanal did not (7).

The use of headspace GC permits the identification of some of the unique volatile aldehydes produced from n-6 and from n-3 PUFA, and allows a more complete comparison of the oxidizability of LDL particles of varying fatty acid composition. This approach thus affords the discriminatory methodology necessary to investigate the effect of LDL composition on the oxidation of lipoprotein particles. An important area that requires further investigation is to determine how the individual aldehydes produced by oxidation of different dietary PUFA react with apo B and how this relates to the atherogenic effects of oxidized LDL particles.

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Superoxide Production by Macrophages Stimulated *in vivo* with Synthetic Ether Lipids

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The anticancer activity of synthetic ether lipids may depend in part upon their ability to activate cells of the monocyte/macrophage lineage. In the present study, we have sought to determine whether 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe) and related ether lipids enhance superoxide production by mouse peritoneal macrophages. Ether lipids were administered intraperitoneally to C57BL/6 mice 4 d after injection with thioglycollate broth. Elicited peritoneal macrophages were harvested and purified one day later, and superoxide production was detected by measuring the superoxide dismutase inhibitable reduction of cytochrome c. Low levels of superoxide were secreted by macrophages in the absence of phorbol 12-myristate 13-acetate (PMA). When PMA was added *in vitro* to macrophages from ET-18-OMe-treated mice, these cells secreted 194.2 nmol superoxide/mg protein in comparison to 53.5 nmol superoxide/mg protein for PMA-treated control cells. The *in vitro* treatment of the macrophages with ET-18-OMe was not effective in stimulating superoxide secretion. Macrophages harvested from mice treated with a series of ether lipids (with and without phosphorus) were examined, and superoxide secretion was found to vary with structure. AM-18-OEt and CP-7 were the most effective compounds, secreting 8.6 and 11.9 times more superoxide, respectively, than PMA-stimulated control cells. Moreover, direct cytotoxicity of the compounds for HL60 human promyelocytic leukemic cells did not necessarily correlate with the ability of each drug to increase macrophage superoxide production.

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Membrane-targeted synthetic ether phospholipids, alkyllysophospholipids (ALP), have been shown to exert antitumor properties by direct cytotoxicity and cytostasis (1–4), by induction of differentiation of malignant cells to the normal phenotype (5,6), and by activation of macrophages to the tumoricidal state (2,7–9). These compounds are currently showing promise as a new class of clinical cancer chemotherapeutic drugs (10–12) and are also in clinical trial as bone marrow purging agents (13).

It has been shown that lysophosphatidylcholine (LPC) accumulates in response to a variety of biological response modifiers and that membrane phospholipids can act as immunomodulators (14,15). Immunomodulation of macrophages may involve activation of phospholipase A_2, resulting in degradation of the membrane phospholipid phosphatidylcholine (PC) to the lyso derivative LPC (2,14). Accordingly, synthetic glycero-3-phosphocholine analogs of LPC were originally designed as stable immunomodulators with longer half-lives than LPC (1,2). These compounds are structural but not functional analogs of LPC and platelet-activating factor, with long-chain ether linkages at position 1 and short-chain ether groups at position 2 of the glycerol backbone.

These agents have been shown to enhance macrophage cytotoxicity (7,16,17) and to activate macrophages (9,18–20). The naturally occurring ether phospholipid, platelet-activating factor, has been shown to stimulate the oxidative burst of guinea pig peritoneal macrophages (21) and to increase glucose consumption in these cells (19). One hallmark of an activated tumoricidal macrophage is the increased production of reactive oxygen metabolites, such as H_2O_2 and superoxide. In this study, the ability of synthetic long-lived ether lipids to stimulate the production of superoxide by mouse peritoneal macrophages was explored as a means of further defining the potential mechanism of anticancer action of this novel class of cancer chemotherapeutic agents.

MATERIALS AND METHODS

Chemicals. The ether lipid ET-18-OMe was kindly provided by Dr. R. Nordström, Medmark Pharma GmbH (Munich, Germany). All other ether lipids were synthesized by Dr. Claude Piantadosi and colleagues, School of Pharmacy, University of North Carolina (Chapel Hill, NC). The structures of the compounds studied are given in Scheme 1. Ether lipid stock solutions were made in ethanol and stored at –20°C, or in phosphate buffered saline (PBS) and sterile filtered immediately prior to use. All other chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Stock solutions included cytochrome c (horse heart type III) dissolved in Hank's balanced salt solution (HBSS) without phenol red to 1.2 mM, phorbol 12-myristate 13-acetate (PMA) solubilized in DMSO to 1 mg/mL and superoxide dismutase (SOD) from bovine erythrocytes dissolved in water at 5 mg/mL.

Animals. Male C57BL/6 mice 4–5 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME).

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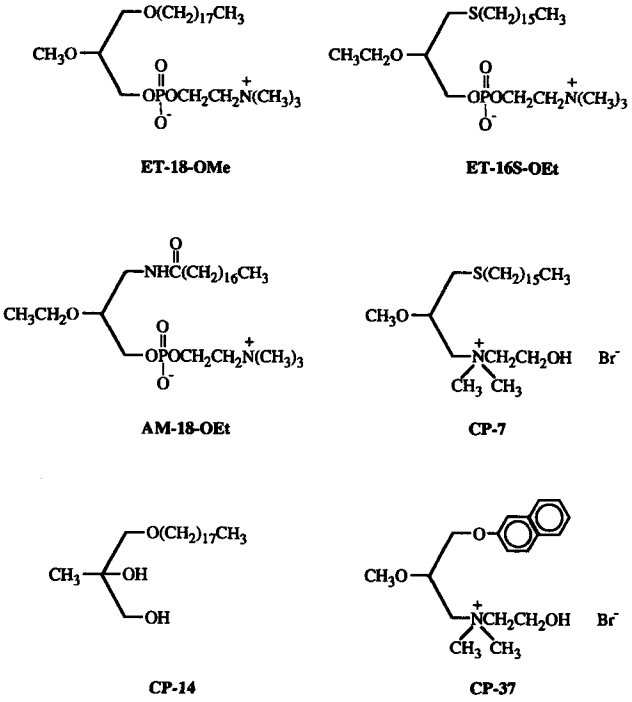
Abbreviations: ALP, alkyllysophospholipids; AM-18-OEt, 1-*N*-heptadecylamido-2-*O*-ethyl-*rac*-glycero-3-phosphocholine; ET-16S-OEt, 1-*S*-hexadecyl-2-*O*-ethyl-*rac*-glycero-3-phosphocholine; ET-18-OMe, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; CP-7, *rac*-1-*S*-hexadecyl-2-*O*-methylthiopropyl-3-*N,N*-dimethyl-β-hydroxyethyl ammonium bromide; CP-14, *rac*-1-*O*-hexadecyl-2-methylglycerol; CP-37, *rac*-1-*O*-β-naphthyl-2-*O*-methylpropyl-3-*N,N*-dimethyl-β-hydroxyethyl ammonium bromide; HBSS, Hank's balanced salt solution; ID_{50}, the amount of compound required to reduce [^{3}H]thymidine incorporation into DNA to 50% of the control value; LPC, lysophosphatidylcholine; MEM, Eagle's minimal essential medium; O_2^-, superoxide; PBS, phosphate buffered saline; PC, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.



SCHEME 1

For *in vivo* activation experiments, mice were injected intraperitoneally with 2 mL of thioglycollate broth. Four days later, intraperitoneal injections (1 mL) of either PBS (control mice) or ether lipid (see Scheme 1) were administered. Twenty-four hours later mice were killed and peritoneal macrophages harvested as described below. For *in vitro* activation experiments, mice were injected intraperitoneally with 2 mL of thioglycollate broth. Four days later mice were killed and peritoneal macrophages harvested as described below.

Macrophage isolation and culture. Peritoneal macrophages were harvested and plated essentially as described by Schreiber *et al.* (22). Mice were killed by cervical dislocation, and macrophages were harvested by peritoneal lavage with 4–5 mL of cold Eagle's minimal essential medium (MEM) supplemented with 2.2 g/L sodium bicarbonate, 1.5× MEM vitamins, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (all purchased from Gibco, Grand Island, NY) and 2 U/mL heparin (Elkins-Sinn Inc., Cherry Hill, NJ). Exudates were pooled for each treatment group (3–5 mice). Cells were centrifuged at 400 × *g* for 10 min, resuspended in MEM, counted and assessed for viability (which was always >95% by trypan blue dye exclusion), and then plated in 24-well tissue culture plates (Corning Glass Works, Corning, NY). Cells were purified by allowing them to adhere to the plastic surface. They were incubated for 2 h at 37°C in an atmosphere of 5% CO_2 in air and then washed three times with HBSS to remove nonadherent cells. By modified Wright's staining (Sigma Diagnostics, St. Louis, MO; according to manufacturer's instructions) and "nonspecific esterase" staining (23), it was determined that the adherent cells consisted primarily of monocytic cells (99%). Cells were incubated in MEM at 37°C in a humidified atmosphere containing 5% CO_2. For *in vivo* activation experiments, superoxide production was then measured; for *in vitro* activation experiments, the cells were then treated with ET-18-OMe prior to determining superoxide production.

Superoxide assay. Superoxide production by purified macrophages was detected by the SOD inhibitable reduction of cytochrome c (24). Cytochrome c stock solutions were diluted in HBSS to 3.5×10^{-4} M and added directly to washed cultures (0.75 mL/well) in the presence or absence of PMA (0.5 μg/mL). Additional cultures also received SOD (40 μg/mL). Cells were incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2 and then placed on ice. The reaction mixtures were transferred to microcentrifuge tubes and were centrifuged at 10,000 × *g* for 5 min at 4°C to remove any cell debris. Spectrophotometric analysis was performed at 550 nm with a Perkin-Elmer spectrophotometer (Palo Alto, CA). Superoxide production was calculated using the extinction coefficient of E_{550} 21.0×10^3 M^{-1} cm^{-1} (25) and expressed as a function of total cellular protein, which was determined as described below.

Protein determinations. After the removal of incubation mixtures from the macrophage cultures, the cells were washed twice with 1.0 mL HBSS, and digested in 0.2 N NaOH overnight at 4°C. Total cellular protein was determined according to Lowry *et al.* (26) with bovine serum albumin as a standard.

Cytotoxicity assay. HL60 human promyelocytic leukemic cells (ATCC, Bethesda, MD) were routinely maintained as suspension cultures in RPMI 1640 medium (Gibco Laboratories) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum (Sigma). Cells were subcultured at 2–3-day intervals and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells in log phase growth were dispensed into 96-well tissue culture plates (Falcon; Becton Dickinson & Co., Lincoln Park, NJ) at 5×10^4 cells/well, and ether lipids were added. Twenty-four hours later, 1.0 μCi/well of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; Dupont NEN Research Products, Boston, MA) was added, and cells were incubated for an additional 24 h. Cells were harvested directly onto glass fiber filters using a MASH II harvester (MA Bioproducts, Walkersville, MD) and counted by liquid scintillation spectroscopy. The ID_{50}, i.e. the amount of compound required to reduce [^{3}H]thymidine incorporation into DNA to 50% of the control value, was determined for each analog tested.

RESULTS AND DISCUSSION

In order to determine the effect of ET-18-OMe on superoxide production by peritoneal macrophages, the SOD inhibitable reduction of cytochrome c was compared in thioglycollate broth elicited cells harvested from control and ET-18-OMe treated mice. Figure 1 shows that macrophages from control animals secreted some superoxide (expressed as a function of cell protein), and the addition of PMA to the cultured cells increased this activity. When macrophages from mice treated intraperi-

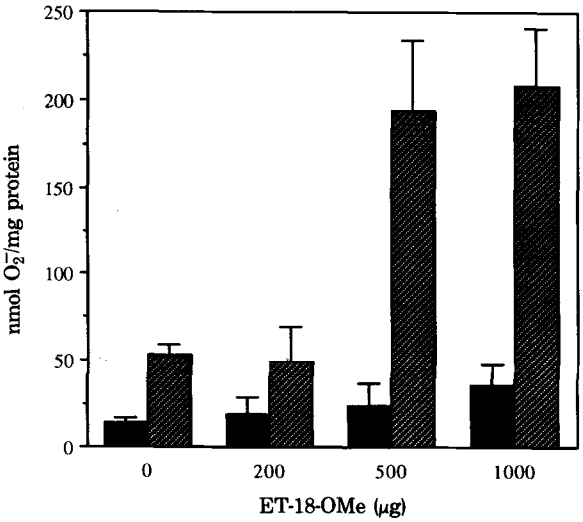


FIG. 1. Superoxide production by PMA-stimulated macrophages from ET-18-OMe-treated mice. Four days after injection of thioglycollate broth, mice were treated with various doses of ET-18-OMe. One day later, peritoneal macrophages were harvested, plated at a density of 5×10^5/well and purified as described in the Materials and Methods section. Superoxide production was determined in the absence (blackened bars) or presence of PMA (hatched bars) as described in Materials and Methods. The values determined from multiple experiments were averaged and the data are expressed as mean ± SEM (n = 7, 3, 5 and 4 for 0, 200, 500 and 1000 μg, respectively).

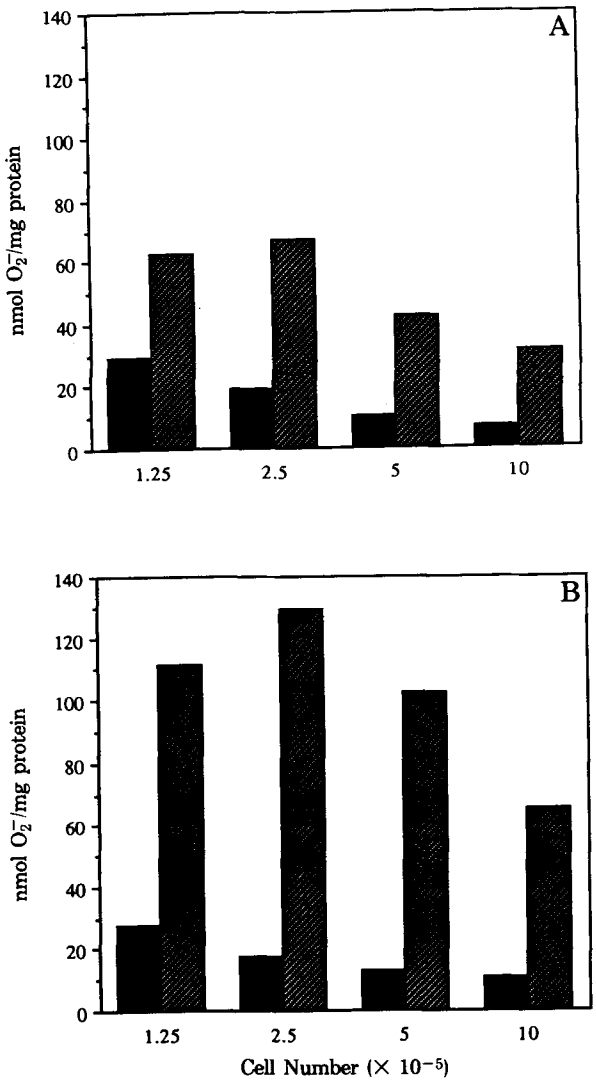


FIG. 2. Superoxide production as a function of macrophage plating density. Four days after injection of thioglycollate broth, mice were treated with either 0 (control; panel a) or 500 μg of ET-18-OMe (panel b). One day later, peritoneal macrophages were harvested, plated at various densities as shown on the X axis, and purified as described in Materials and Methods. Superoxide production was determined in the absence (blackened bars) or presence of PMA (hatched bars) as described in Materials and Methods. The data from a representative experiment are expressed as mean of duplicate determinations.

toneally with various doses of ET-18-OMe were assessed, there were small increases in activity in comparison to control cells. However, when PMA was added *in vitro* to the macrophages from mice treated with 500 or 1000 μg ET-18-OMe, there was a substantial increase in superoxide. The PMA-induced increase was apparent at the time the cells were isolated from the animal as shown, as well as 2 h later, although the stimulation decreased by 24 h later (data not shown). For all subsequent studies, the dose of 500 μg was used.

It has previously been shown (27) that the amount of superoxide produced per cell decreases as cell numbers increase. Therefore, the effect of plating density on superoxide production by macrophages from ET-18-OMe treated mice was examined. Figure 2A shows that as the number of cells plated from control mice increased, superoxide production on a per mg protein basis decreased; however, PMA stimulation was apparent at all cell concentrations. Likewise, Figure 2B shows that at the highest plating density of cells from ET-18-OMe treated mice, the levels of superoxide were lowest, but once again the PMA-induced increase was apparent.

In order to determine whether ET-18-OMe can increase superoxide production by macrophages treated with the drug *in vitro,* cells were harvested 4 d after injection with thioglycollate broth. The cells were purified as for the previous experiments and then treated with ET-18-OMe for various periods of time. In this manner, the cell population that was treated *in vitro* with the drug was similar to that treated *in vivo,* i.e., the cells elicited 4 d after treatment of the mice with thioglycollate broth. Figures 3A and 3B show the effect of treatment with ET-18-OMe for 2 h and 24 h, respectively, on superoxide production. As shown before, the PMA-induced increase was apparent; however, no significant differences were noted between control untreated cells and drug treated cells. Higher concentrations of ET-18-OMe (10 μM) were toxic to the cell cultures; in comparison to control cultures, levels of cell protein were 70 and 30% in the presence of the drug after 2 and 24 h, respectively. It therefore appears that at least under the conditions tested, *in vitro* stimulation to the activated state was not possible and that activation required additional stimuli encountered *in vivo* such as soluble

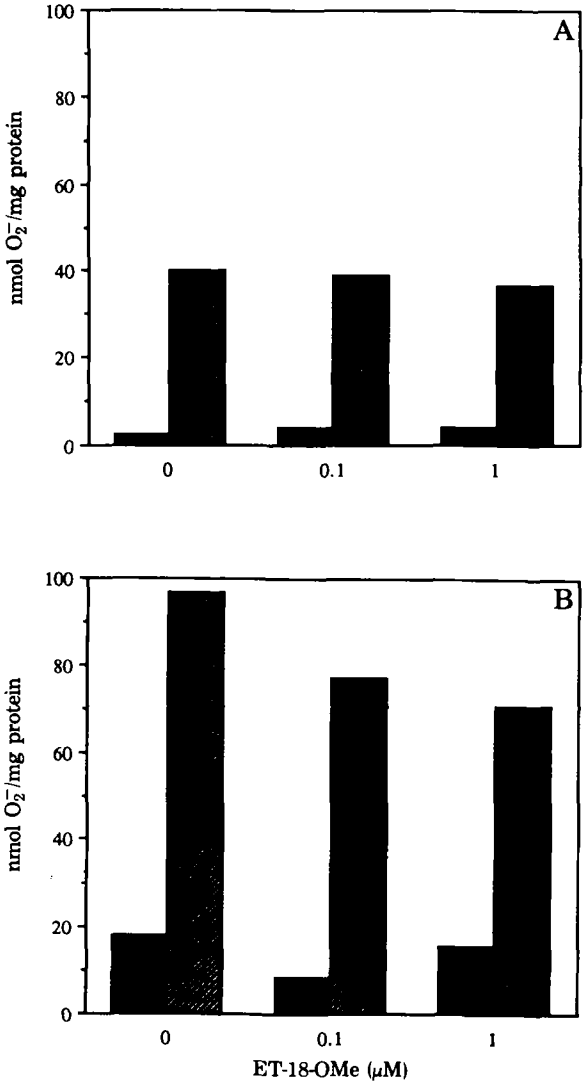


FIG. 3. Superoxide production by macrophages treated *in vitro* with ET-18-OMe. Four days after injection of thioglycollate broth, peritoneal macrophages were harvested, and purified as described in Materials and Methods. After 2 h (panel a) or 24 h (panel b) treatments *in vitro* with ET-18-OMe at the indicated concentration, superoxide production was determined in the absence (blackened bars) or presence of PMA (hatched bars) as described in Materials and Methods. The data from a representative experiment are expressed as mean of duplicate determinations.

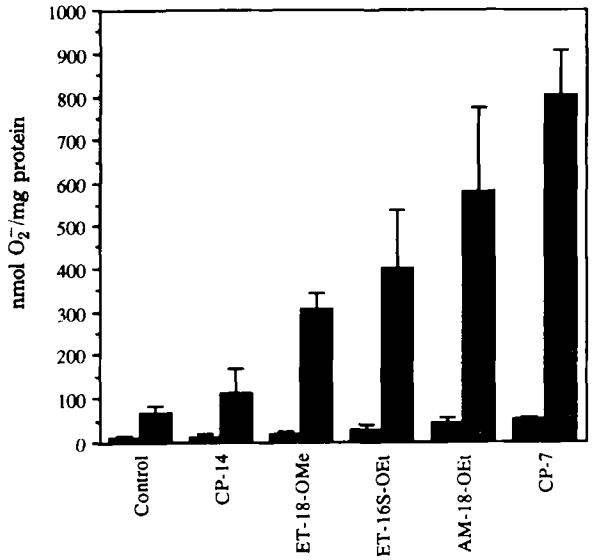


FIG. 4. Superoxide production by PMA-stimulated macrophages from mice treated with various ether lipids. Four days after injection of thioglycollate broth, mice were either sham-treated (control) or treated with 500 μg of CP-14, ET-18-OMe, ET-16S-OEt, AM-18-OEt or CP-7. One day later, peritoneal macrophages were harvested, plated at a density of 2.5×10^5/well and purified as described in Materials and Methods. Superoxide production was determined in the absence (blackened bars) or presence of PMA (hatched bars) as described in Materials and Methods. The values determined from multiple experiments were averaged, and the data are expressed as mean ± SEM (n = 3 for CP-14, ET-16S-OEt, AM-18-OEt and CP-7; n = 4 for control and ET-18-OMe).

mediators or lymphocytes. Similarly, Talmadge *et al.* (9) found that macrophage-mediated cytotoxicity for mouse melanoma B16-BL6 target cells was apparent only when the ether lipid was administered *in vivo*.

The studies on ET-18-OMe were extended to examine the effect of a variety of synthetic ether lipids (structures given in Scheme 1) on superoxide production. In this series of experiments, mice that had received thioglycollate broth 4 d earlier were treated with 500 μg of the appropriate compound. Twenty-four hours later mice were killed, peritoneal macrophages harvested and superoxide production evaluated in the presence and absence of PMA. The data in Figure 4 show that some of the compounds stimulated superoxide production, which was further enhanced by PMA treatment. Macrophages displayed a wide range of activity depending upon the initial drug treatment. Superoxide production by cells from mice treated with CP-37 (data not shown) and CP-14 was approximately equivalent to controls, in that there was no stimulation. ET-18-OMe and ET-16S-OEt displayed a "mid" range of activity and yet others, AM-18-OEt and CP-7, were 8.6 and 11.9 times, respectively, more active than the PMA-stimulated control cells.

Table 1 shows the direct cytotoxicity to HL60 tumor cells of the drugs tested above. In an assay in which HL60 cells were exposed to the drugs for 48 h, the dose at which [^{3}H]thymidine incorporation into DNA was 50% of the control value was determined. The data show that the compounds that were most highly cytotoxic to tumor cells were not necessarily the most potent macrophage activators.

Undoubtedly, regulation of the generation of reactive oxygen metabolites is critical to maintaining the balance between the positive role these products play in antimicrobial and antitumor activities and the potential negative effects they may have in promoting tissue damage. It has been shown that a number of agents including LPS (28) and cytokines such as interferon-γ (29–33) can "prime" macrophages for an enhanced respiratory burst. We refer to the effect noted here as "priming," which implies persistence of the cells' response to the initial agent (ET-18-OMe) even after its removal; however, we

TABLE 1

The Direct Cytotoxicity of Synthetic Ether Lipids to HL60 Cells[a]

Compound	ID_{50} (μM)
ET-16S-OEt	2.19 ± 0.44 (3)[b]
ET-18-OMe	2.38 ± 0.40 (21)[b]
AM-18-OEt	3.30 ± 0.37 (5)[b]
CP-7	4.34 ± 0.55 (5)[b]
CP-14	19.06 ± 1.82 (6)
CP-37	>100

[a]HL60 cells were exposed to the compounds for 48 h (the last 24 h of which was in the presence of [^{3}H]thymidine). ID_{50} represents the dose (μM) at which [^{3}H]thymidine incorporation into DNA was 50% of the control value. Each experiment was performed in quadruplicate. The average for each experiment (number of experiments listed in parentheses) was used to determine mean ± SD.

[b]These values were previously reported by Morris-Natschke *et al.* (36).

cannot rule out a synergistic effect mediated by a potential interaction of PMA with residual ET-18-OMe. If this were the case, however, *in vitro* treatment of the macrophages with the ether lipid would be expected to have resulted in stimulation of superoxide production. Although there was no statistically significant difference between cell yields from sham-treated vs. drug-treated mice (data not shown), the possibility that drug treatment has selected for a population of cells *in vivo* that is more responsive to a PMA-induced respiratory burst still exists.

Although it is generally accepted that PMA action involves activation of protein kinase C activity, ET-18-OMe inhibits the activity of this enzyme (6,34). Others have suggested that ether lipids exert a biphasic effect on protein kinase C, stimulating the enzyme at low concentrations and causing inhibition at higher concentrations (2,35). Clearly, more work is needed to determine the mechanism(s) by which ether lipid "primed" macrophages respond to PMA with an enhanced respiratory burst, or how "primed" macrophages may be cytotoxic to tumor cells *in vivo*.

Our studies demonstrate that various synthetic ether lipids have the potential to stimulate macrophage superoxide secretion. Both phosphorus and nonphosphorus containing compounds show macrophage activating properties. The tumoricidal activity of any of these ether lipids may result from a combination of its direct cytotoxicity and its tumoricidal macrophage activating potential. Although the direct cytotoxicity by these compounds is undoubtedly the major contribution to their tumoricidal capacity, these studies reinforce that they can potentially activate tumoricidal macrophages as well. Since the ability of a particular analog to activate tumorigenic macrophages does not necessarily correlate with direct tumor cytotoxicity, designs for effective therapeutic modalities may necessitate inclusion of a combination of ether lipids.

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Modification of Lysine Amino Groups by the Lipid Peroxidation Product 4,5(*E*)-Epoxy-2(*E*)-heptenal

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The reaction between 4,5(*E*)-epoxy-2(*E*)-heptenal (EH) and L-lysine was studied to characterize some of the compounds that may be produced when proteins react with peroxidizing lipids. A mixture of EH and lysine was incubated overnight at room temperature and then fractionated by high-performance liquid chromatography (HPLC). Fractions were freeze-dried and characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectrometry. Four major pyrrole derivatives were obtained, namely 1-(5'-amino-1'-carboxypentyl)-pyrrole (3), 1-(5'-amino-1'-carboxypentyl)-2-(1"-hydroxypropyl)pyrrole (diastereomers 5 and 8), 1-(5'-amino-5'-carboxypentyl)pyrrole (7), and 1-(5'-amino-5'-carboxypentyl)-2-(1"-hydroxypropyl)pyrrole (9). In addition, several lysine complexes were detected. A polymer (1b) that was responsible for the color and the fluorescence produced in the reaction was isolated by gel filtration chromatography from a fraction obtained by HPLC. Formation of pairs of analogs (5 and 3, 9 and 7) with and without a substituent in position 2 of the pyrrole ring suggested that the compounds were produced by the same mechanism, with the formation of the 2-unsubstituted pyrroles corresponding to the loss of the 2-substituent as propanal; propanal was detected by headspace capillary gas chromatography. A reaction mechanism is proposed based on the NMR data obtained when the reaction was monitored in real time in an NMR tube. The results suggest that pyrrolic amino acids 7 and 9 may be present in proteins that have been damaged by peroxidizing lipids.

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The reactions of free radicals, such as peroxyl and hydroxyl radicals, with biomolecules are important in physiology and pathology (1). Lipid peroxidation is the result of free radical mediated reactions that occur *in vivo* in aerobic organisms (2) and is known to be accompanied by the formation of a complex mixture of products, including aldehydes (3). Among these aldehydes, the formation of 4,5-epoxy-2-alkenals, mainly 4,5(*E*)-epoxy-2(*E*)-heptenal (EH) and 4,5(*E*)-epoxy-2(*E*)-decenal, has been reported (4,5). The products are assumed to be formed by cleavage of the carbon chain of intermediary epoxyhydroperoxy fatty acids (6). Thus, for example, 12,13(*E*)-epoxy-9-hydroperoxy-10(*E*)-octadecenoic acid, which was identified in the reaction of 13-hydroperoxylinoleic acid with either soybean extracts or ferric chloride/cysteine solution (7), produced the C_{10} epoxyaldehyde 4,5(*E*)-epoxy-2(*E*)-decenal.

EH is a product of the oxidation of ω3 pentaenoic fatty acids (8). EH has also been detected in homogenates from fish (Zamora and Hidalgo, unpublished results) that have a high content of ω3 eicosapentaenoic acid. The reaction of EH with amines and amino acids very rapidly develops color and fluorescence similar to that of lipofuscin-like fluorescent substances (9). The mechanism of color and fluorescence formation has been investigated for the reaction of EH with butylamine and glycine methyl ester which produced 1-alkyl-2-(1'-hydroxyalkyl)pyrroles. The color and fluorescence produced in this polymerization reaction was characterized (10).

The objective of the present study was to identify and characterize the reaction products formed from EH and lysine to extend the results obtained with simple amines to lysine, the amino acid that is usually lost in the course of protein deterioration caused by peroxidizing lipids (11). The complete characterization of these products should provide the basis for the development of an analytical method that can measure the formation of these products in damaged proteins.

MATERIALS AND METHODS

EH was prepared from 2(*E*)-4(*E*)-heptadienal as described by Swoboda and Peers (4). 2(*E*)-4(*E*)-heptadienal, L-lysine monohydrochloride and 3-chloroperoxybenzoic acid were purchased from Aldrich Chemical Co. (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 instrument (Karlsruhe, Germany) using sodium-3-(trimethylsilyl)-1-propanesulfonate as internal standard. Two-dimensional NMR was used to assign ^{13}C NMR spectra. Mass spectra were obtained on an AEI-MS-30SB-VG mass spectrometer (VG Analytical, Manchester, United Kingdom). Gel filtration chromatography (GFC) was performed on a Sephadex G-25 (Pharmacia LKB, Uppsala, Sweden) column (55 × 1 cm) equilibrated with 50 mM sodium phosphate, pH 7. Fractions (900 μL) were collected at a flow rate of 9 mL/h, and tested for absorption at 430 nm, and for fluorescence with excitation at 390 nm and emission at 450 nm. The column was calibrated with dextran sulfate [molecular weight (MW) 5000], bacitracin (MW 1423) and glucotropaeoline potassium salt (MW 447.5). Assays were also done on a Biogel P-2 (Bio-Rad Laboratories, Hercules, CA) column of similar dimensions.

Reaction of EH with lysine. 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

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Abbreviations: EH, 4,5(*E*)-epoxy-2(*E*)-heptenal; GC/MS, gas chromatography/mass spectrometry; GFC, gel filtration chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MW, molecular weight; NMR, nuclear magnetic resonance.

room temperature. The reaction mixture was fractionated by semipreparative high-performance liquid chromatography (HPLC) on a C18 column (25 × 1 cm) using 5% acetonitrile in deionized water as eluent at 40°C. The flow rate was 5 mL/min with detection of absorbance at 250 nm. Products were collected from successive injections, and combined. Solutions were dried in a stream of nitrogen to remove acetonitrile, and were freeze-dried. The compounds isolated were characterized by ^{1}H and ^{13}C NMR and by mass spectrometry (MS).

Headspace capillary gas chromatography (GC) detection of propanal. Propanal was detected by GC/MS. Samples of EH (8 mg, 64 μmol) and lysine monohydrochloride (23.8 mg, 130 μmol) in 0.3 M sodium borate buffer pH 9.0 (500 μL) were introduced into special 10-mL headspace bottles, which were sealed with silicone rubber and Teflon caps and incubated overnight at room temperature. Thereafter, samples were pressurized for 30 s before injection. GC/MS analyses were done on a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph interfaced, *via* an open coupling system, to an AEI-MS-30SB-VG mass spectrometer. A DB-5 bonded-phase fused-silica capillary column (JW Scientific, Folsom, CA) kept at 30°C was used, and helium served as carrier gas. Standard propanal (Aldrich) was used for identification purposes.

NMR study of the EH / lysine reaction. EH (0.16 mmol) was suspended in 1 mL of deuterated water and sonicated with a Braun Labsonic U sonicator (B. Braun Diessel Biotech GmbH, Melsungen, Germany) until completely emulsified. Lysine (0.32 mmol) was then added, and the resulting solution was introduced into a 5-mm NMR tube. The pH of the solution was taken to slightly basic by adding a few drops of deuterated sodium hydroxide, and ^{1}H and ^{13}C NMR spectra were taken at different time points. Zero time was considered the time before adding alkali.

RESULTS

Reaction of EH with lysine. The reaction between EH and lysine rapidly produced color and fluorescence (9). A characteristic chromatogram of the reaction mixture after overnight incubation is shown in Figure 1. Fourteen fractions were isolated, and the structures of the components were determined by ^{1}H and ^{13}C NMR, and by MS. When a fraction consisted of only one compound, the fraction number was also used as compound number. When the fraction consisted of two compounds, each compound was identified by the number of the fraction, followed by a letter. Figure 2 shows the structures of the compounds identified in this study.

Fraction **1** (474.3 mg, 87.4%) was composed of a mixture that was subsequently fractionated by GFC. It consisted of unreacted lysine (compound **1a**, more than 95% of the fraction) and a polymer (**1b**) that was responsible for the color and fluorescence produced in the reaction. Figure 3 shows the fluorescence spectra of the reaction mixture and of fraction **1** and of the polymer **1b** isolated by GFC. The spectra did not change in the course of the purification. The spectra also were similar to those produced by the lipofuscin-like fluorescent substances that we described earlier (10). Polymer **1b** eluted in the void volume of the Biogel P-2 column, which has a fractionation range of MW 100–1800. The chromatographic elution pattern of polymer **1b** from Sephadex G-25 is shown in Figure 4. The polymer showed an apparent MW of 1900 daltons.

Fraction **2** (1.6 mg, 0.3%) was unstable, but could be characterized by ^{1}H NMR. Its spectrum showed signals at δ 8.25 *d* (*J* = 17 Hz) and 8.17 *d* (*J* = 12 Hz) and was tentatively identified as an olefin derived from EH and lysine. The spectral integrals suggested an EH/lysine adduct ratio of 1:1. Additional studies will be necessary for the complete characterization of fraction **2**.

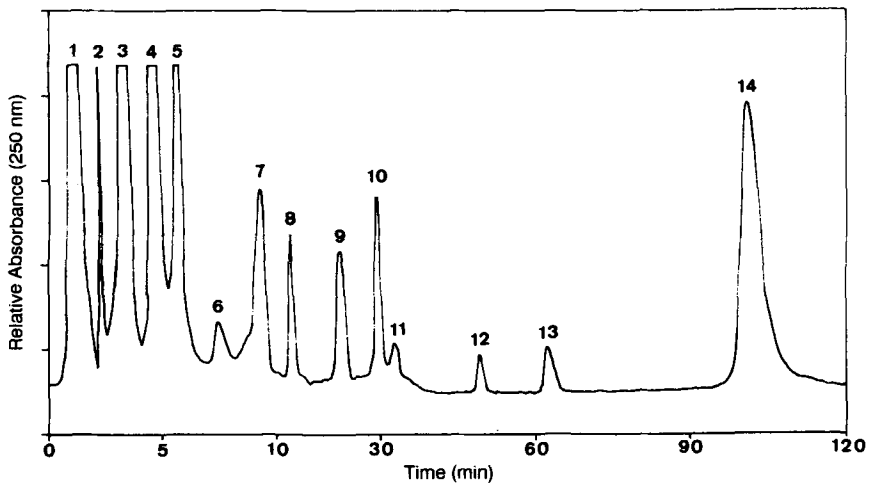
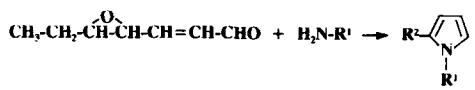


FIG. 1. High-performance liquid chromatogram of the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction after stirring overnight at room temperature.



Compound	R^1	R^2
1a	$(CH_2)_4$-CH(NH_2)COOH	
3	CH(COOH)-$(CH_2)_4NH_2$	H
5	CH(COOH)-$(CH_2)_4NH_2$	CH(OH)CH_2CH_3
7	$(CH_2)_4$-CH(NH_2)COOH	H
8	CH(COOH)-$(CH_2)_4NH_2$	CH(OH)CH_2CH_3
9	$(CH_2)_4$-CH(NH_2)COOH	CH(OH)CH_2CH_3
15	CH_3	CH(OH)CH_3

FIG. 2. Structures of the compounds isolated from the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction by high-performance liquid chromatography and characterized by ^1H and ^{13}C nuclear magnetic resonance and mass spectrometry.

Fraction **3** (7.1 mg, 1.3%) was identified as 1-(5'-amino-1'-carboxypentyl)pyrrole (**3**). ^1H NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 1.30 *m* (2H, H-3'), 1.67 *m* (2H, H-4'), 2.06 *m* (2H, H-2'), 2.94 *t* (2H, $J_{5',4'}$ = 7.6 Hz, H-5'), and 4.52 *dd* (1H, *J* = 5.8 Hz and *J* = 9.8 Hz, H-1'). ^{13}C NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 25.4 *t* (C-3'), 29.0 *t* (C-4'), 34.6 *t* (C-2'), 42.0 *t* (C-5'), 67.1 *d* (C-1'), and 181.7 *s* (COOH). The mass spectrum (70 eV) *m/e* (%, ion structure) was as follows: 196 (2, M^+), 178 (83, $M^+ - H_2O$), 152 (48, $M^+ - CO_2$), 135 (37, 152 – NH_3), 120 (59), 106 (42), 94 (71, ethylpyrrole – H), 80 (76, methylpyrrole – H), 67 (100, pyrrole). MS (70 eV) of the methyl ester obtained by reaction of **3** with diazomethane, *m/e* (%, ion structure): 210 (59, M^+), 193 (16, $M^+ - NH_3$), 178 (11, $M^+ - CH_3OH$), 151 (68, $M^+ - CO_2CH_3$), 134 (100, 151 – NH_3).

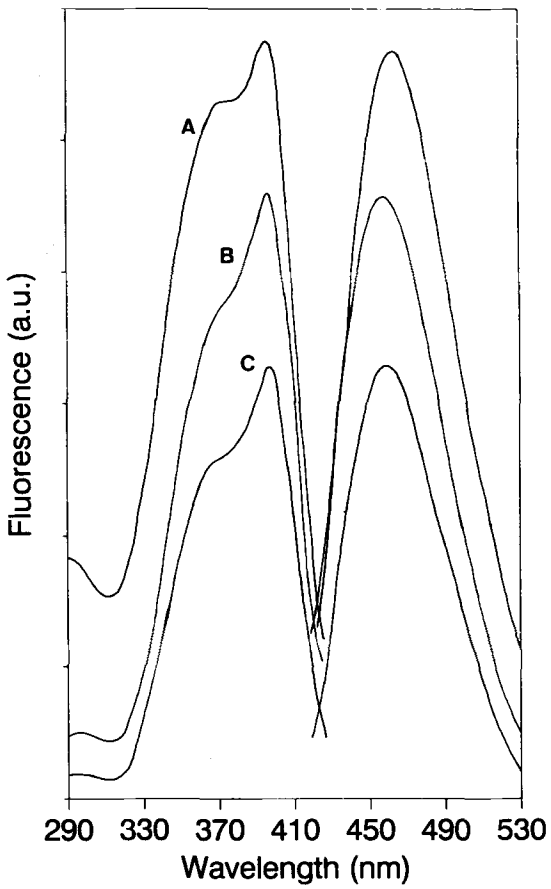


FIG. 3. Fluorescence spectra of the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction mixture (A), fraction 1 (B) and polymer 1b (C).

Fraction **4** (3.8 mg, 0.7%) was unstable and its structure could not be determined. The ^1H spectrum showed signals at δ 7.41 *t* (1H, *J* = 7.8 Hz), 7.52 *dq* (1H, *J* = 7.8 Hz, *J* = 1.1 Hz, *J* = 1.1 Hz, *J* = 1.1 Hz), 7.54 *dt* (1H, *J* = 7.8 Hz, *J* = 1.1 Hz, *J* = 1.1 Hz) and 7.82 *t* (1H, *J* = 1.1 Hz); the ^{13}C NMR spectrum showed signals at d 129.0, 130.6, 132.9 and at 131.7 ppm, suggesting a heterocyclic structure, most probably due to a pyridinium salt. Proton and carbon signals corresponding to lysine were also present. Additional studies are necessary for the complete characterization of a fraction **4**.

Fraction **5** (2.3 mg, 0.4%) was identified as 1-(5'-amino-1'-carboxypentyl)-2-(1"-hydroxypropyl)pyrrole (**5**). ^1H NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 0.91 *t* (3H, $J_{2",3"}$ = 7.4 Hz, H-3"), 1.36 *m* (2H, H-3'), 1.69 *m* (2H, H-4'), 1.86 *m* (2H, H-2"), 2.13 *m* (2H, H-2'), 2.94 *t* (2H, $J_{4',5'}$ = 7.3 Hz, H-5'), 4.56 *t* (1H, $J_{1",2"}$ = 7.0 Hz, H-1") and 4.72 *dd* (1H, *J* = 5.7 Hz, *J* = 9.6 Hz, H-1'). ^{13}C NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 12.7 *q* (C-3"), 25.6 *t* (C-3'), 29.1 *t* (C-4'), 31.5 *t* (C-2"), 34.2 *t* (C-2'), 42.0 *t* (C-5'), 63.5 *d* (C-1'), 69.4 *d* (C-1") and 181.8 *s* (COOH). MS (70 eV) *m/e* (%, ion structure): 236 (7, $M^+ - H_2O$), 192 (7, $M^+ - H_2O - CO_2$), 134 (38, 192 – $C_3H_6NH_2$), 84 (100, tetrahydropyridine + H^+). MS (70 eV) of the methyl ester obtained by reaction with diazomethane, *m/e* (%, ion structure): 236 (7, M^+ – MeOH), 218 (21, $M^+ - H_2O$ – MeOH), 179 [27, 1-methoxycarbonylmethyl-2-(1-propenyl)pyrrole], 134 [42, 1-ethenyl-2-(1-propenyl)pyrrole + H^+], 108 [34, 2-(1-propenyl)pyrrole + H^+], 84 (100, tetrahydropyridine + H^+).

Fraction **6** (1.5 mg, 0.3%) was a minor compound and only could be studied by ^1H NMR. The spectrum showed heterocyclic proton signals at δ 6.17, 6.79 and 6.82 ppm, suggesting the presence of a 1,2-disubstituted pyrrole ring. The spectrum also showed the signals corresponding to the lysine protons, and the signal corresponding to proton H-1" (the alcohol produced upon epoxide ring opening) appeared at δ 4.55 *t* (1H, *J* = 6.8 Hz). Additional analyses will be needed for the complete characterization of **6**.

Fraction **7** (8.0 mg, 1.5%) was identified as 1-(5'-amino-5'-carboxypentyl)pyrrole (**7**). ^1H NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 1.34 *qu* (2H, *J* = 6.8 Hz, H-3'), 1.83 *m* (4H, H-2' and H-4'), 3.68 *t* (1H, $J_{4',5'}$ = 6.1 Hz, H-5'), and 3.97 *t* (2H, $J_{1',2'}$ = 6.9 Hz, H-1'). ^{13}C NMR (D_2O) showed

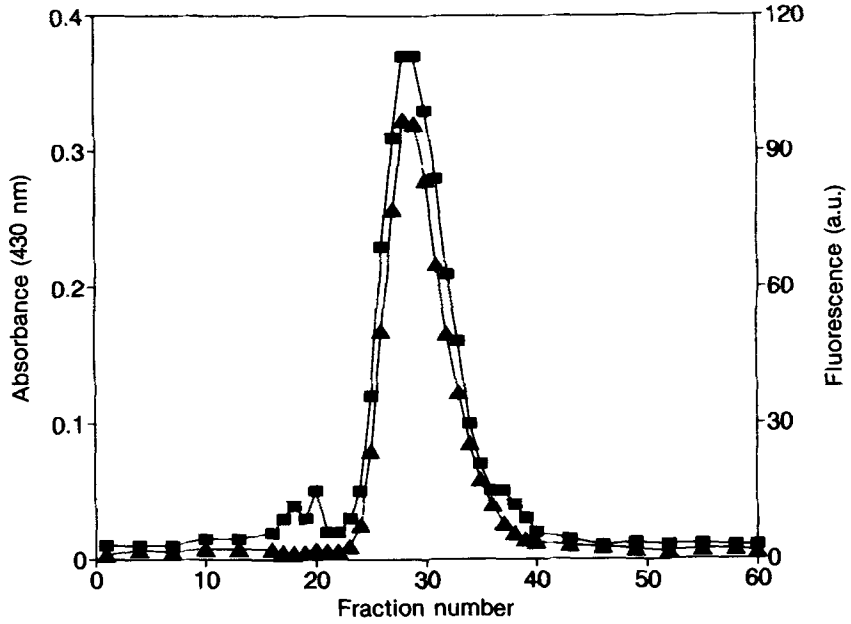


FIG. 4. Gel filtration chromatography of fraction 1 on a Sephadex G-25 column. Absorbance at 430 nm (■) and fluorescence at 390/450 nm (▲) were measured for each fraction. Void and total volumes corresponded to fractions 20 and 48, respectively.

the pyrrole signals listed in Table 1. Other signals appeared at δ 24.4 *t* (C-3'), 33.0 *t* and 33.2 *t* (C-2' and C-4'), 51.4 *t* (C-1'), 57.6 *d* (C-5') and 181.4 *s* (COOH). MS (70 eV) *m/e* (%, ion structure): 196 (61, M^+), 178 (20, $M^+ - H_2O$), 152 (16, $M^+ - CO_2$), 134 (58, 152 – NH_4), 122 (47, $M^+ - C_2H_4NO_2$), 109 (27, propylpyrrole), 94 (37, ethylpyrrole – H), 81 (100, methylpyrrole). MS (70 eV) of the methyl ester obtained by reaction with diazomethane, *m/e* (%, ion structure): 210 (45, M^+), 178 (22, $M^+ - CH_3OH$), 134 (100, $M^+ - CO_2CH_3 - NH_3$), 122 (26, butylpyrrole – H), 108 (15, propylpyrrole – H), 80 (68, methylpyrrole – H).

Fraction **8** (4.9 mg, 0.9%), in analogy to fraction **5**, was found to be 1-(5'-amino-1'-carboxypentyl)-2-(1"-hydroxypropyl)pyrrole (**8**). ^1H NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 0.91 *t* (3H, $J_{2",3"}$ = 7.4 Hz, H-3"), 1.38 *m* (2H, H-3'), 1.67 *m* (2H, H-4'), 1.86 *m* (2H, H-2"), 2.13 *m* (2H, H-2'), 2.92 *t* (2H, $J_{4',5'}$ = 7.5 Hz, H-5'), 4.62 *t* (1H, $J_{1",2"}$ = 7.1 Hz, H-1"), and 4.71 *dd* (1H, *J* = 5.6 Hz and *J* = 9.9 Hz, H-1'). ^{13}C NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 12.8 *q* (C-3"), 25.6 *t* (C-3'), 29.2 *t* (C-4'), 31.4 *t* (C-2"), 34.6 *t* (C-2'), 42.0 *t* (C-5'), 63.8 *d* (C-1'), 70.0 *d* (C-1") and 181.5 *s* (COOH).

TABLE 1

^1H NMR Chemical Shifts and Coupling Constants (in Hz) and ^{13}C NMR Chemical Shifts of the Pyrrole Moieties of the Compounds Produced in the EH/Lysine Reaction

Compound	H-2	H-3	H-4	H-5	C-2	C-3	C-4	C-5
3	6.84*t* *J* = 2.1	6.18*t* *J* = 2.1	6.18*t* *J* = 2.1	6.84*t* *J* = 2.1	123.5*d*	110.3*d*	110.3*d*	123.5*d*
5	—	6.17*m*	6.17*m*	6.90*t* *J* = 2.2	137.9*s*	108.0*d*[a]	109.4*d*[a]	122.3*d*
7	6.85*t* *J* = 1.9	6.18*t* *J* = 1.9	6.18*t* *J* = 1.9	6.85*t* *J* = 1.9	124.3*d*	110.2*d*	110.2*d*	124.3*d*
8	—	6.16*m*	6.16*m*	6.87*t* *J* = 2.3	137.9s	108.4*d*[a]	109.6*d*[a]	123.0*d*
9	—	6.18*dd*[b]	6.13*t* *J* = 3.1	6.81*t* *J* = 2.1	137.3*s*	108.0*d*	109.2*d*	125.0*d*
15[c]	—	6.02*dd*[d]	5.92*dd*[d]	6.55*dd*[d]	136.5s	106.4*d*	107.0*d*	123.6*d*

[a]Assignments may be interchanged. [b]$J_{3,5}$ = 1.8 Hz, $J_{3,4}$ = 3.4 Hz. [c]Synthesis of compound **15** was described previously (10). Spectra were taken in $(CD_3)_2CO$, and Me_4Si was used as internal standard. [d]$J_{3,5}$ = 1.5 Hz, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ = 2.9 Hz.

MS (70 eV) *m/e* (%, ion structure): 236 (6, $M^+ - H_2O$), 192 (15, $M^+ - H_2O - CO_2$), 134 (40, 192-$C_3H_6NH_2$), 84 (100, tetrahydropyridine + H^+). MS (70 eV) of the methyl ester obtained by reaction with diazomethane, *m/e* (%, ion structure): 268 (3, M^+), 236 (11, M^+ – MeOH), 218 (8, $M^+ - H_2O$ – MeOH), 179 [30, 1-methoxycarbonylmethyl-2-(1-propenyl)pyrrole], 134 [38, 1-ethenyl-2-(1-propenyl)pyrrole + H^+], 108 [18, 2-(1-propenyl)pyrrole + H^+], 84 (100, tetrahydropyridine + H^+). Compounds **5** and **8** had analogous ^1H and ^{13}C NMR spectra, as well as mass spectra. The only significant difference observed between the spectra was the proton H-1" chemical shift that was characteristic for each compound. Compounds **5** and **8** are considered to be the diastereomers [1'(*S*),1"(*R*) and 1'(*S*),1"(*S*)]. Compound **9** (see next paragraph) should be a similar mixture of diastereomers; however, these isomers could not be separated under our conditions.

Fraction **9** (7.5 mg, 1.4%) was identified as 1-(5'-amino-5'-carboxypentyl)-2-(1"-hydroxypropyl)pyrrole (**9**). ^1H NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 0.93 *t* (3H, $J_{2",3"}$ = 6.7 Hz, H-3"), 1.36 *m* (2H, H-3'), 1.79 *m* (4H, H-2' and H-4'), 1.88 *m* (2H, H-2"), 3.62 *t* (1H, $J_{4',5'}$ = 6.0 Hz, H-5'), 3.97 *m* (2H, H-1'), and 4.65 *t* (1H, $J_{1",2"}$ = 6.9 Hz, H-1"). ^{13}C NMR (D_2O) showed the pyrrole signals listed in Table 1. Other signals appeared at δ 12.8 *q* (C-3"), 24.5 *t* (C-3'), 31.7 *t* (C-2"), 33.4 *t* (C-2' or C-4'), 33.4 *t* (C-4' or C-2'), 48.6 *t* (C-1'), 57.5 *d* (C-5'), 69.3 *d* (C-1"), and 181.1 *s* (COOH). MS (70 eV) *m/e* (%, ion structure): 236 (9, $M^+ - H_2O$), 218 (13, 236 – H_2O), 192 (11, $M^+ - H_2O - CO_2$), 134 (40, 192 – $C_3H_6NH_2$), 84 (100, tetrahydropyridine + H^+). MS (70 eV) of the methyl ester obtained by reaction with diazomethane, *m/e* (%, ion structure): 268 (8, M^+), 250 (43, $M^+ - H_2O$), 191 (22, $M^+ - H_2O - CO_2CH_3$), 174 (53, 191 – NH_3), 162 (47, 250 – $CH(NH_2)CO_2CH_3$), 134 [100, 1-ethenyl-2-(1-pro-penyl)-pyrrole + H^+], 120 [45, 1-methyl-2-(1-propenyl)pyrrole – H), 84 (53, tetrahydropyridine + H^+).

Fractions **10** (4.9 mg, 0.9%), **11** (6.5 mg, 1.2%), **12** (7.7 mg, 1.4%), and **13** (7.1 mg, 1.3%) gave ^1H and ^{13}C NMR spectra very similar to those of lysine, and are therefore undefined complexes between lysine molecules and some lipid ligands. Fraction **14** (5.4 mg, 1.0%) was unstable and decomposed after concentration. No spectral data could be obtained on this fraction.

Detection of propanal. The formation of pairs of analogous compounds (**5** and **3**, **9** and **7**) with and without a substituent in position 2 of the pyrrole ring suggested that the compounds were produced by the same mechanism. According to the mechanism proposed for this reaction (see below), loss of the substituent at position 2 would imply the formation of propanal. Indeed, propanal was detected by GC/MS after incubation of the reaction mixture in a special headspace bottle. Identification of the aldehyde was confirmed by comparison with standard propanal.

NMR study of the EH/lysine reaction. An initial ^1H NMR spectrum (Fig. 5A) was acquired before the reaction mixture was treated with deuterated sodium hydroxide, and it showed the additive spectra of EH and lysine. Signals were assigned as follows. Signals for EH: A (H-1), B (H-3), C (H-2), F (H-4), G (H-5), K (H-6) and M (H-7). Signals for lysine: E (H-2), H (H-6), I (H-3), J (H-5) and L (H-4). Signal D was due to water. Addition of a few drops of NaOD to the mixture initiated the reaction causing rapid spectral changes (Fig. 5B). The aldehyde (A) and epoxy (F and G) proton signals rapidly

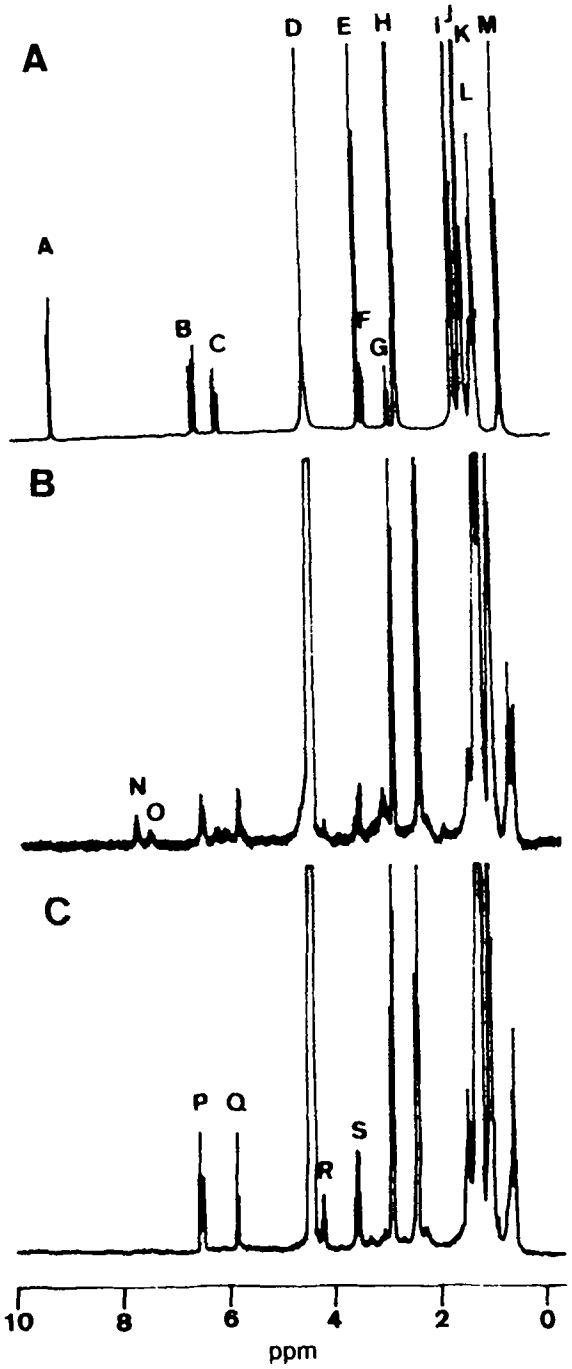


FIG. 5. Changes produced in the ^1H nuclear magnetic resonance spectrum of the 4,5(*E*)-epoxy-2(*E*)-heptenal/lysine reaction with incubation time: A, initial spectrum of the mixture without NaOD; B, spectrum taken at 2–4 min after adding a few drops of NaOD; C, spectrum taken after 2 h.

disappeared, the olefinic proton signals were transformed (B and C), and new signals appeared. Some of the signals corresponded to intermediates (see, for example, signals N and O), which again disappeared later. The final spectrum (Fig. 5C), which remained unchanged after 2 h into the reaction, showed at least four new signals (P, Q, R and S) that were not present in the original spectrum, and suggested that the epoxy and olefin protons were altered. The number of scans for consecutive spectra was increased with incubation time to maintain similar signal-to-noise ratios. Formation of polymers was consistent with observed signal loss and was also suggested by the increase in brown color with incubation time. Similar results were also obtained by ^{13}C NMR (spectra not shown), with the final spectrum showing the appearance of carbon signals at δ 105–130 ppm, consistent with the presence of heterocyclic carbons.

DISCUSSION

The reaction of EH with lysine is very rapid, produces a brown color, as well as fluorescence within a short period of time. When the reaction was followed by NMR in a sample tube, EH aldehydic, olefinic and epoxy protons disappeared after a few minutes (Fig. 5). The final spectrum (Fig. 5C) was quite simple and suggested the presence of only a few products. However, when the mixture was fractionated by HPLC, 14 fractions were isolated to be further studied by NMR and MS. Five of these fractions (fractions **3, 5, 7, 8** and **9**) corresponded to pyrrole monomers that were produced from one molecule of EH linked to one amino group of lysine. These five fractions were the major products, with the exception of fraction **1**, that included excess lysine remaining in the reaction. The sum of the spectra of these fractions was very similar to final spectrum of the reaction (Fig. 5C). A possible mechanism for the pyrrole formation is suggested in Figure 6. It implies the formation of an imine at a first stage that would produce the pyrrole ring by intramolecular attack of the nitrogen at one of the epoxy carbons. The departure of a proton, which may be accompanied by formation of propanal, would produce the pyrroles isolated. This mechanism is also in agreement with the detection of propanal by headspace GC, and with the results obtained when EH/lysine reaction was monitored by ^{1}H and ^{13}C NMR. Thus, signals N and O in Figure 5B may be assigned to the imine proton of the first intermediate, and the final spectrum (Fig. 5C) showed the presence of the pyrrolic monomers corresponding to signals P, Q, R and S.

Other fractions (fractions **2, 4** and **6**) from the EH/lysine reaction mixture also corresponded to single structure with olefinic bonds (compound **2**) or heterocyclic rings (compounds **4** and **6**). The olefinic and heterocyclic proton signals of compounds **2** and **4** (δ ≈ 8.2 and ≈ 7.5 ppm, respectively) were not detected in the reaction spectrum (Fig. 5C), suggesting that they were minor compounds in the reaction.

That polymerization occurred in this reaction was consistent with the ^{1}H and ^{13}C NMR spectra taken on the reaction mixture where the number of scans had to

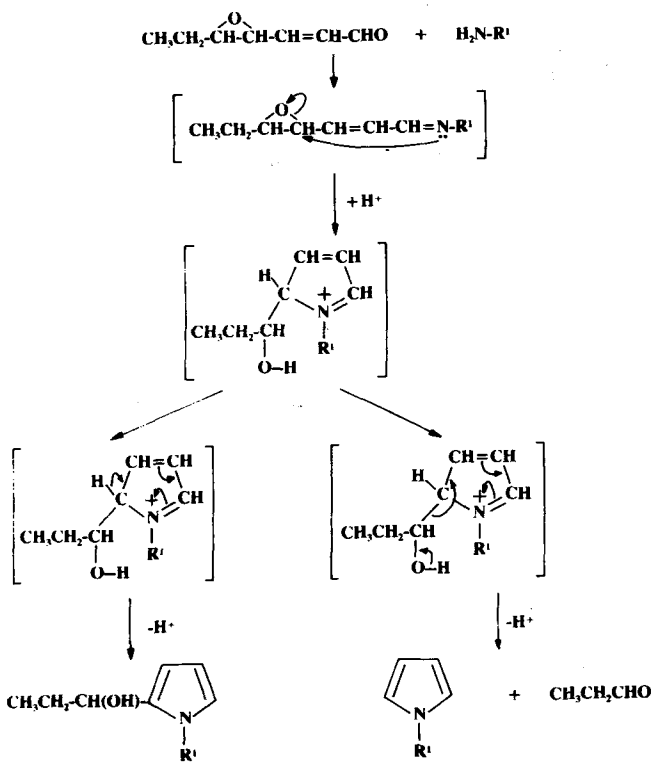


FIG. 6. Proposed mechanism of pyrrole formation in the reaction of 4,5(*E*)-epoxy-2(*E*)-heptenal with lysine.

be increased with incubation time to obtain analogous signal-to-noise ratios. The polymer appeared in fraction **1** together with the unreacted lysine. The polymer was responsible for the color and the fluorescence produced in the reaction and had an apparent MW of 1900 daltons. This result is very similar to that expected for a polypyrrolic structure, as described previously (10), and is consistent with a structure of this type for polymer **1b.** The results obtained in this study confirm that the reaction between EH and lysine at pH 9.0, or under physiological conditions (12), follows an analogous mechanism and consequently produces compounds analogous to those formed in previously described model reactions between EH and butylamine or glycine methyl ester (10). The pyrrole monomers **3, 5, 7, 8** and **9,** may therefore be produced *in vivo,* and additional studies are needed to confirm their formation. Pyrrole monomers **7** and **9** are the only ones of the pyrroles described that can be formed with the ε-amino group of lysine and, accordingly, may be present in protein hydrolysates. However, the other pyrroles might also be produced by reaction with the existing pool of free amino acids. Further studies of the formation of pyrrole amino acids with proteins should reveal the role of epoxyaldehydes in the overall modification of proteins by lipid peroxidation products.

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Incorporation of *trans* Long-Chain n-3 Polyunsaturated Fatty Acids in Rat Brain Structures and Retina[1]

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During heat treatment, polyunsaturated fatty acids and specifically 18:3n-3 can undergo geometrical isomerization. In rat tissues, 18:3 Δ9*c*,12*c*,15*t*, one of the *trans* isomers of linolenic acid, can be desaturated and elongated to give *trans* isomers of eicosapentaenoic and docosahexaenoic acids. The present study was undertaken to determine whether such compounds are incorporated into brain structures that are rich in n-3 long-chain polyunsaturated fatty acids. Two fractions enriched in *trans* isomers of α-linolenic acid were prepared and fed to female adult rats during gestation and lactation. The pups were killed at weaning. Synaptosomes, brain microvessels and retina were shown to contain the highest levels (about 0.5% of total fatty acids) of the *trans* isomer of docosahexaenoic acid (22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*). This compound was also observed in myelin and sciatic nerve, but to a lesser extent (0.1% of total fatty acids). However, the ratios of 22:6 *trans* to 22:6 *cis* were similar in all the tissues studied. When the diet was deficient in α-linolenic acid, the incorporation of *trans* isomers was apparently doubled. However, comparison of the ratios of *trans* 18:3n-3 to *cis* 18:3n-3 in the diet revealed that the *cis* n-3 fatty acids were more easily desaturated and elongated to 22:6n-3 than the corresponding *trans* n-3 fatty acids. An increase in 22:5n-6 was thus observed, as has previously been described in n-3 fatty acid deficiency. These results encourage further studies to determine whether or not incorporations of such *trans* isomers into tissues may have physiological implications.

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During heat treatment of oils and fats, geometrical isomerization of linolenic (1,2) and linoleic (3) acids occurs. Three major isomers of linolenic acid (18:3 Δ9*c*,12*c*,15*t*; 18:3 Δ9*t*,12*c*,15*c* and 18:3 Δ9*t*,12*c*,15*t*) are formed, as well as some minor isomers. The *trans* compounds were identified as well in commercial frying oils (4), in deodorized oils (1) and in low-calorie spreads (5). Little is known about the metabolism and physiological effects of these *trans* isomers of linolenic acid (6,7). However, a recent study (8) has indicated that the intestinal absorption of these compounds is similar to that of linolenic acid.

Furthermore, 18:3 Δ9*c*,12*c*,15*t*, an isomer of linolenic acid, could undergo further desaturation and elongation to yield *trans* isomers of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, i.e., 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* (20:5 Δ17*t*) and 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t* (22:6 Δ19*t*). These isomers were identified in the liver of rats fed heated linseed oils (9) and were found to be incorporated into almost all lipid classes, including phospholipids (10).

It is possible that these compounds can also be incorporated into tissues rich in n-3 polyunsaturated fatty acids (PUFA), such as retina or certain brain structures (synaptosomes, capillaries and microvessels). The nervous system is, indeed, rich in structural lipids, and the essential character of the n-3 fatty acids for the development and function of the brain and retina is now well-documented (11). Moreover, some other nervous structures, such as myelin and sciatic nerve, are known to have an apparently lower turnover of their fatty acids (12) and seem better protected than other organs against n-3 deficiency (13). It was the purpose of the present work to determine whether *trans* PUFA would be incorporated into brain structures using diets adequate or deficient in α-linolenic acid.

MATERIALS AND METHODS

Preparation of concentrates of trans *fatty acids.* The geometrical isomers of α-linolenic acid were prepared by two methods that were previously described (14). In the first method, a purified fraction (99%) of methyl α-linolenate was obtained from linseed oil after urea inclusion and preparative high-performance liquid chromatography (HPLC). This fraction was then isomerized with nitrous acid, and the 18:3 isomers were purified by column chromatography. The second method uses linseed oil that was heated for 12 h at 275°C under nitrogen. The fatty acid methyl esters (FAME) obtained from the heated oil were then purified by column chromatography. The cyclic monomers were removed by urea inclusion, and the geometrical isomers of α-linolenic acid were concentrated by preparative HPLC. The composition of the two fractions is detailed in Table 1. The two fractions, which were the methyl esters, were then transformed into ethyl esters by saponification (batch of 300 g) and subsequent esterification with ethanol by refluxing in the presence of 0.5% sulfuric acid for 5 h under nitrogen.

Animals and diets. All studies were done within the guidelines set for the care and use of laboratory animals, in agreement with official French regulations (authorizations 3273 and A 21100). Wistar rats from Iffa-Credo (L'Arbresle, France) were fed for two generations with a

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

Delta nomenclature (Δ) is used for *trans* polyunsaturated fatty acids to specify the position and geometry of ethylenic bonds. Polyunsaturated fatty acids containing *trans* double bonds are abbreviated giving the locations of the *trans* double bonds only; e.g., 20:5 Δ17*t*, 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t*; 22:5 Δ19*t*, 22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t*; 22:6 Δ19*t*, 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*.

TABLE 1

Fatty Acid Composition of the Two Fractions Containing *trans* Isomers of Linolenic Acid[a]

Fatty acid	Fractions[b] A	B
16:0	—	10.0
18:0	—	7.4
18:1 Δ9*t*	0.1	0.9
18:1 Δ9*c*	0.3	27.3
18:2 Δ9*t*,12*t*	0.1	0.4
18:2 Δ9*c*,12*t*	0.5	4.9
18:2 Δ9*t*,12*c*	0.6	4.9
18:2 Δ9*c*,12*c*	1.7	12.6
Conjugated 18:2 *c,t*[c]	0.1	0.2
Conjugated 18:2 *t,t*[d]	0.1	0.7
18:3 Δ9*t*,12*t*,15*t*	1.2	0.3
18:3 Δ9*t*,12*c*,15*t*	2.9	10.9
18:3 Δ9*c*,12*t*,15*t*	4.1	1.5
18:3 Δ9*t*,12*t*,15*c*	3.0	1.8
18:3 Δ9*c*,12*c*,15*t*	12.4	5.8
18:3 Δ9*c*,12*t*,15*c*	12.5	3.5
18:3 Δ9*t*,12*c*,15*c*	9.6	4.3
18:3 Δ9*c*,12*c*,15*c*	50.3	1.3
Unknowns	0.3	1.2

[a]Values are means of at least three determinations. Each fatty acid is expressed as percent of the total fraction.

[b]The purified fractions containing the *trans* isomers of linolenic acid were prepared as follows: Fraction A was obtained by isomerization of pure methyl α-linolenate, using nitrous acid and the 18:3 isomers were then purified by column chromatography. For fraction B, fatty acid methyl esters obtained from a heated linseed oil (275°C, 12 h, under nitrogen) were purified by column chromatography, urea inclusion and preparative high-performance liquid chromatography.

[c]*cis,trans* and *trans,cis* conjugated linoleic acid.

[d]*trans,trans* conjugated linoleic acid.

TABLE 2

Composition of the Diets of the Female Rats During Mating, Gestation and Lactation

	Groups A	B, C, D
	g/kg diet	g/kg diet
Casein	205	205
DL-Methionine	5	5
Starch	427	425
Saccharose	277	276
Lipids[a]	16	19
Agar-agar	20	20
Mineral mixture[b]	50	50
Vitamin mixture[c]	+	+

[a]Group A received 5 g/kg diet of fraction A obtained by chemical isomerization of α-linolenate and 11 g/kg diet of sunflower oil ethyl esters. Group B received 10 g/kg diet of fraction B extracted from heated linseed oil and 9 g/kg diet of sunflower oil ethyl esters. Groups C and D received 19 g/kg diet of the same blend of soybean, peanut and linseed oils (35:44:21), but group C received it as ethyl esters and group D as triglycerides.

[b]Composition of the mineral mixture (g/kg of diet): $CaHPO_4$, 10; $CaCO_3$, 10; KH_2PO_4, 12.5; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 5; 4 $MgCO_3$/Mg $(OH)_2 \cdot 5\ H_2O$, 2; oligoelements (mg/kg of diet): $FeSO_4 \cdot 7\ H_2O$, 500; $MnSO_4 \cdot H_2O$, 250; $ZnSO_4 \cdot 7H_2O$, 300; $CuSO_4 \cdot 5\ H_2O$, 60; $KAl(SO_4)_2 \cdot 12\ H_2O$, 10; NaF, 5; $NiSO_4 \cdot 7\ H_2O$, 5; $CoSO_4 \cdot 7\ H_2O$, 5; K_2CrO_4, 5; KIO_3, 2; $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 1; NH_4VO_3, 0.5; Na_2SeO_3, 0.3.

[c]Vitamin mixture was prepared in water-soluble form (except for inositol and *para*-aminobenzoic acid) and was added each day to water, lipids and dry diet to provide the semi-liquid diet. The composition was as follows (per kg dry diet): DL-α-tocopheryl acetate, 100 mg; choline chloride, 1 g; biotin, 0.2 mg; folic acid, 2 mg; thiamin · HCl, 10 mg; riboflavin, 10 mg; pyridoxine · HCl, 10 mg; pantothenic acid, 30 mg; niacin, 60 mg; vitamin B_{12}, 0.05 mg; ergocalciferol (in alcohol), 1000 IU; *trans* retinol palmitate (water dispersable), 6000 IU; phytomenadione, 1 mg; inositol, 200 mg; *para*-aminobenzoic acid, 100 mg.

pellet diet (T87) containing only 320 mg n-3 fatty acids per k/g of diet (15). This diet, which contained (in g/100 g) wheat 22, casein 17.5, methionine 0.5, skim milk powder 10, brewer's yeast 5, corn starch 27, cellulose 4, sunflower oil 10, and mineral and vitamin blend 4, was pelleted by Fabrique de Mélanges Expérimentaux (Inra, La Minière, France).

Ninety-six female adult rats of the pellet-fed animals were mated and fed the experimental diets (Table 2) during mating, gestation and lactation. The purified semi-liquid diet was prepared fresh daily by blending dry diet, water, vitamin solution and experimental lipids. The animals were fed *ad libitum*. Four groups of 24 rats each (A, B, C and D) were used. The groups differed only by the type of lipids in their diet. Groups A and B were the experimental groups and received the two fractions containing linolenic acid *trans* isomers. Group A received 5 g/kg diet of the first fraction obtained by chemical isomerization of α-linolenic acid (Table 1, A) and group B 10 g/kg diet of the second fraction extracted from heated linseed oil (Table 1, B). Sunflower oil ethyl esters were added to these two diets to provide a sufficient quantity of linoleic acid (11 and 9 g/kg diet, respectively). Groups C and D were the control groups. They were fed the same diet containing 19 g/kg of a blend of soybean, peanut and linseed oils (35:44:21, by wt) as lipids. However, group D received this blend as triglycerides, while group C received it as ethyl esters. The esters were obtained from the blend by saponification and subsequent esterification, as previously described. These two control groups were necessary because the isomer fractions were obtained as fatty acids. Dietary lipids are usually present mostly as triglycerides, and some studies suggest that the intestinal absorption and metabolism of PUFA are not necessarily the same for the triglycerides, ethyl esters and fatty acids (16).

The quantitites of the different sources of lipids were chosen to arrive at (i) equivalent quantities of linoleic acid in the four groups, (ii) equivalent levels of all *cis* α-linolenic acid in groups A, C and D, and (iii) comparable levels of the desaturable *trans* isomer of α-linolenic acid (18:3 Δ9*c*,12*c*,15*t*) in groups A and B (Table 3). The quantities of total n-3 fatty acids (*cis* + *trans*) were similar in groups B, C and D, and the total 18:3 *trans* isomers in groups A and B differed only by about 20%. However, it was not possible to have the same level of total lipids in group A compared to the other groups (16 vs. 19 g/kg); compensation was achieved by adding 1 g/kg of saccharose and 2 g/kg of starch (Table 2).

Large litters of animals were reduced to eight pups at birth. The survival of pups, the food intake of dams, and

TABLE 3

***Trans* Fatty Acids and Essential Fatty Acids in the Diets[a]**

Fatty acid	Groups A	B	C and D
	mg/kg diet		
18:2 Δ9*c*,12*c*	7961	7704	7452
18:3 Δ9*c*,12*c*,15*c*	2526	139	2705
18:3 Δ9*c*,12*c*,15*t*	620	580	—
Total 18:3 *trans* isomers	2285	2810	—
Total n-3 fatty acids	4811	2949	2705
18:2 Δ9*c*,12*c*/18:3 Δ9*c*,12*c*,15*c* ratio	3.15	55.42	2.75
18:3 Δ9*c*,12*c*,15*t*/total n-3 ratio	0.129	0.197	—
18:3 Δ9*c*,12*c*,15*t*/(18:3 Δ9*c*,12*c*,15*t* + 18:3 n-3) ratio	0.197	0.807	—
Total *trans* 18:3/total n-3 fatty acids ratio	0.475	0.953	—

[a]Values are means of at least three determinations.

the weight of the litters and of the dams were recorded. The pups were killed at weaning by decapitation. After exsanguination, the brain, sciatic nerve, retina and liver were dissected and removed. Myelin and nerve endings (synaptosomes) were obtained, and their purity was checked as described previously (17).

Brain capillaries were separated according to Goldstein (18), and their purity was checked as described previously (19). All fractions were lyophilized. After dissection, the livers of the weanling rats were immediately placed in a mixture of chloroform/methanol (2:1, vol/vol).

Fatty acid analyses. The liver lipids were extracted according to Folch *et al.* (20), and the lipids of the lyophilized preparations were obtained using the method of Pollet *et al.* (21). The lipids were transmethylated with BF_3/methanol according to Metcalfe and Schmitz (22). The FAME were freed from dimethylacetals by thin-layer chromatography (TLC) on silica plates (ref. 5720; Merck, Darmstadt, Germany) using toluene as solvent, as described by Morrison and Smith (23). The structures of the dimethylacetals were verified by gas–liquid chromatography (GLC) coupled with mass spectrometry.

The FAME were analyzed by GLC. GLC analyses were carried out on a Becker-Packard 417 or 420 (Middleburg, The Netherlands), fitted with a solid injector, or on a Carlo Erba Mega 5300 (Milano, Italy), equipped with an on-column injector. All chromatographs utilized flame-ionization detection. Fused silica capillary columns were used, principally a CP Sil 88 column (Chrompack, Middleburg, The Netherlands) 50 m long, internal diameter 0.32 mm, film thickness 0.25 μm. Other columns used were a DB 23 and a DB Wax (J&W Scientific, Rancho Cordo, CA), 30 m long, internal diameter 0.25 mm, film thickness 0.25 μm. The analyses were done isothermally at 175 or 185°C, or programmed from 50 to 200°C at 15°C/min. The temperature of both the injector and detector was 240°C. Helium was the carrier gas. Quantitative data were obtained using Vista CDS 401 (Varian, Les Ulis, France) or Chromjet (Spectraphysics, Les Ulis, France) integrators.

Fatty acids were identified by comparison with commercial standards (Nu-Chek-Prep, Elysian, MN) whenever possible. In addition, 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* and 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*, which we previously prepared and identified (9), were used as GLC standards. FAME of identical preparations were also pooled and then separated by $AgNO_3$–TLC for further identification. Silica gel plates (Merck; 0.25 mm layers, 20 cm × 20 cm) were impregnated by immersing them for 30 min into a solution of $AgNO_3$ in acetonitrile (0.59 mol/L). The developing solvent was toluene/diethyl ether (90:10, vol/vol). The bands were visualized after spraying with a 0.2% solution of 2'7'-dichlorofluorescein in ethanol under ultraviolet light at 54 nm and then were scraped into glass centrifuge tubes. Release of the unsaturated fatty acids from the $AgNO_3$ adsorbent was facilitated by washing with NaCl in methanol/water following a modification (2) of the method of Hill *et al.* (24). FAME were also analyzed by GLC on different columns. Finally, some structures (saturated, mono- and di-ethylenic FAME, dimethylacetals) were also checked by GLC coupled with mass spectrometry. A Hewlett-Packard 5970 Mass Selective Detector (Palo Alto, CA) coupled to a Hewlett-Packard 5890 gas chromatograph was used. A DB Wax fused silica column (30 m long, internal diameter 0.25 mm, film thickness 0.5 μm; J&W Scientific) was employed in the splitless injection mode. The temperature was programmed from 50 to 200°C at 20°C/min, held at 200°C for 45 min, then programmed from 200 to 220°C at 20°C/min, and finally held at 220°C until completion of the analysis. Helium was the carrier gas.

Statistical anallysis. Food intake of the dams during pregnancy, the number of live pups by litter and the weight of the pups at day 19 were analyzed by analysis of variance. The differences in weight of live pups at birth were calculated using the analysis of covariance, the covariable being the total number of pups born (alive and stillborn). Fetuses born dead and postnatal mortality were compared using analysis of variance on angular transforms of proportions, according to Gabriel (25). In all cases, the effect of *trans* isomers (A,B vs. C,D), linolenic acid deficiency (B vs. A,C,D) and ethyl esters (D vs. A,B,C) were calculated.

Concerning the fatty acid composition of brain structures, only three pools were available in each group (but only one pool for retina), and it was not possible to efficiently calculate the significance of differences for these conditions.

RESULTS AND DISCUSSION

The reproduction characteristics of the different groups were quite similar (Table 4), and no effects of the *trans* fatty acids or of the form of administration (triglyceride vs. fatty acid ethyl esters) were detected under our experimental conditions. The dietary deficiency in *cis* α-linolenic acid was also not sufficient to induce an increase in perinatal mortality, as already observed (26). The apparent higher level of postnatal mortality in group C was produced principally in one litter, and the effect was not significant. Only one significant effect could be seen in the growth of the young, namely that the weights of the rats fed the diet containing triglycerides as lipid source (group D) was significantly higher than those of the rats

TABLE 4

Reproduction Characteristics and Growth of Rats Fed *trans* Isomers of Linolenic Acid

	Groups				Effect of analysis of variance[a]		
	A	B	C	D	*Trans* isomers	18:3n-3 deficiency	Ethyl esters
Number of experimental female rats	24	24	24	24			
Number of litters born	12	16	17	16			
Food intake of dams during pregnancy (g dry matter)[b]	578.4 ± 31.8	553.5 ± 41.0	565.8 ± 41.7	578 ± 35.3	NS	NS	NS
Live pups/litter	9.92	11.75	10.94	9.94	NS	NS	NS
Weight of live pups at birth (g)[c]	5.92	5.67	5.49	5.65	NS	NS	NS
Fetuses born dead (as % of total pups born)[d]	1.65	2.08	3.12	1.24	NS	NS	NS
Postnatal mortality (from birth to day 3)[d]	4.76	3.23	9.3	3.33	NS	NS	NS
Number of litters weaned	12	16	16	15	—	—	—
Weight of young of the litter of eight pups at day 19 (g)[b]	43.17 ± 1.88	42.08 ± 3.86	41.94 ± 5.51	46.95 ± 2.53	NS	NS	**

[a]Analyses of variance and covariance were done as described in the Materials and Methods section. The following factors were tested: *trans* isomers = (A,B vs. C,D); linolenic acid deficiency = (B vs. A,C,D); ethyl ester = (D vs. A,B,C). NS, not significant ($P > 0.05$); **significant for $P < 0.001$.

[b]Values are means ± SE.

[c]Significance of differences calculated using covariance with number per litter.

[d]Significance of differences calculated using analysis of variance on angular transformations.

receiving the ethyl esters. However, this effect did not impair the comparison of the composition of lipid fatty acids between groups.

The fatty acid composition of brain structures was determined by at least three chromatographic analyses on CP Sil 88 phase and ascertained by GLC on two other phases, as well as by isolating samples by $AgNO_3$–TLC. These analyses made it possible to detect differences in the retention times of *trans* long-chain fatty acids compared to normal *cis* fatty acids on the three GLC phases (Fig. 1). The equivalent chain lengths of some of the important PUFA on carbowax 20 M column had been previously measured (9). The CP Sil 88 column, which affords the same type of separation as the carbowax 20 M column, was more efficient. The separation of the same compounds on the DB 23 phase is different as the two *trans* isomers of 22:5n-3 and 22:6n-3 have a shorter retention volume than their *all cis* homologs. These particularities proved very useful for the identification of these compounds in the various lipid fractions. With the very small quantities available it was not possible to check for the presence of the *trans* ethylenic bond by GLC coupled with Fourier transform infrared spectroscopy.

The fatty acid composition of the nerve endings (synaptosomes) is presented in Table 5. The control groups C and D had fatty acid compositions similar to those described previously (17). The synaptosomes from group A contained a lower level of 22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t*, the *trans* isomer of 22:5n-3, and a higher level of 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t*, the *trans* isomer of 22:6n-3. It is important to note that the incorporation of such *trans* compounds apparently did not affect the levels of the *cis* n-3 or n-6 long-chain PUFA, which were similar to those of the control groups. If we compare the level of 18:3 Δ9*c*,12*c*,15*t* in the diet (19.7% of the desaturable 18:3; see Table 3) with the level of *trans* 22:6 in synaptosomes [0.49/(15.65 + 0.49) = 3% of total 22:6], it is obvious that there is less deposition of *trans* 22:6 than of *cis* DHA in synaptosomes. *Trans* isomers of α-linolenic or EPA were also not detected. This observation confirms some preliminary earlier results (10) and suggests that these *trans* PUFA are present in the lipid classes or tissues in which their corresponding *cis* analogs are usually found.

It could be hypothesized that the incorporation of *trans* PUFA would be higher in lipids of animals of group B that were slightly deficient in α-linolenic acid, and this was, in effect, the case. The level of 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t* in group B was more than twice that in group A. However, this increase was not sufficient to compensate for the decrease in 22:6n-3. As has been observed in other cases of linolenic acid deficiency (17,27,28), the level of 22:5n-6 was higher than that in group A and in the controls. Not even traces of 22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t* were detectable in group B. Moreover, the level of 22:5n-3 decreased. It seems that all the n-3 fatty acids available were further elongated toward the final compounds of the series.

In brain capillaries and microvessels (Table 6) and in retina (Table 7), the results were similar, except that no

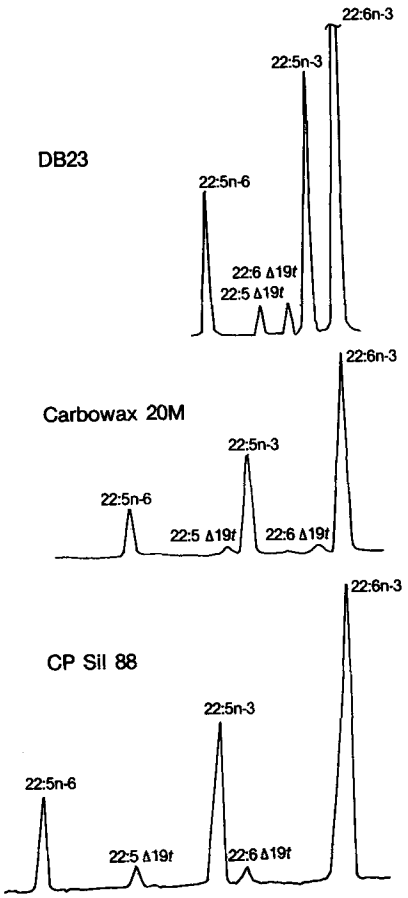

FIG. 1. Gas–liquid chromatograms of some *cis* and *trans* docosapentaenoic and docosahexaenoic acids (standards were prepared and characterized by Grandgirard *et al.*, Ref. 9). The three GLC phases used were DB23 (J&W Scientific, Rancho Cordo, CA), Carbowax 20M (J&W, Palo Alto, CA) and CP Sil 88 (Chrompack, Middleburg, The Netherlands). The chromatographic conditions used are described in Materials and Methods section.

22:5 Δ7*c*,10*c*,13*c*,16*c*,19*t* was detected in brain microvessels of group A and that a small quantity of this compound was found in the retina of group B. Moreover, in retina of group B not only 22:5n-6 but also 22:4n-6 and 20:4n-6 increased. However, due to the very low weight of retina, only one pool was available in each group.

Myelin and sciatic nerve have fatty acid compositions different from those of synaptosomes, brain microcapillaries and retina. Myelin and sciatic nerve are rich in saturated and monoenic fatty acids and relatively low in PUFA (12,17,29). It is generally thought that the fatty acids of myelin and sciatic nerve have relatively long turnover times. However, under our experimental conditions, small, but relevant, quantities of *trans* n-3 PUFA were incorporated into the tissues of group A (Tables 8 and 9). The level of 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t* was approximately doubled in myelin and tripled in sciatic nerve of group B compared to group A. As has already been reported for other tissues, this increase was not sufficient to compensate for the large decrease in 22:6n-3. The levels of 22:5n-6 were increased as in other brain structures and in retina. However, in sciatic nerve, also some other changes in fatty acid composition were observed in group B. The 22:4n-6 and 20:4n-6 levels apparently increased, while linoleic acid levels decreased. In this case, it is possible that the formation of long-chain n-6 PUFA was depressed by the n-3 fatty acids in group A and that the significant decrease in n-3 levels in group B allowed linoleic acid to be desaturated and elongated to a much greater extent.

All brain tissues studied contained the *trans* 22:6n-3 isomer when *trans* isomers of linolenic acid had been part of the diet. When *all cis* dietary α-linolenic acid was sufficient (group A), the levels of this *trans* 22:6n-3 isomer ranged from 0.08 to 0.64% of total FAME. However, if the incorporation of *trans* long-chain PUFA is calculated as the percentage of DHA that was of *trans* geometry, it becomes evident that the incorporation of *trans* DHA is similar (from 2.6 to 3.7%) in all the brain structures (Table 10).

TABLE 5

Fatty Acid Composition of Synaptosomes[a]

Fatty acids	Groups			
	A	B	C	D
Saturated	41.26 ± 0.85	43.10 ± 0.28	41.26 ± 0.54	41.40 ± 0.14
Monoenoic	15.03 ± 1.44	15.88 ± 0.13	16.46 ± 0.22	16.35 ± 0.13
n-6 PUFA				
18:2	1.01 ± 0.03	1.01 ± 0.02	1.17 ± 0.09	1.13 ± 0.04
20:3	0.53 ± 0.06	0.36 ± 0.04	0.50 ± 0.07	0.44 ± 0.02
20:4	16.23 ± 0.36	17.33 ± 0.15	17.22 ± 0.54	16.69 ± 0.23
22:4	3.45 ± 0.09	3.75 ± 0.19	3.28 ± 0.11	3.48 ± 0.33
22:5	1.92 ± 0.10	10.89 ± 0.35	2.27 ± 0.27	1.89 ± 0.18
n-3 PUFA				
22:5 Δ19*t*	0.10 ± 0.01	—	—	—
22:5	0.30 ± 0.04	0.18 ± 0.05	0.30 ± 0.06	0.27 ± 0.03
22:6 Δ19*t*	0.49 ± 0.05	1.13 ± 0.12	—	—
22:6	15.65 ± 0.48	4.20 ± 0.31	15.32 ± 0.11	16.62 ± 0.40
Others	3.05 ± 0.51	2.17 ± 0.22	2.22 ± 0.20	1.72 ± 0.19

[a]Values are % of total fatty acids and are means ± SE of three pools. Synaptosomes were obtained from pups at weaning and were lyophilized. After extraction of the lipids and methylation, fatty acid methyl esters were analyzed by gas–liquid chromatography on three columns: CP Sil 88, DB 23 and DB Wax. The chromatographic conditions and the procedures for peak identification are given in the Materials and Methods section. PUFA, polyunsaturated fatty acid.

TABLE 6

Fatty Acid Composition of Brain Capillaries and Microvessels[a]

Fatty acids	Groups			
	A	B	C	D
Saturated	42.54 ± 0.26	44.22 ± 0.12	43.72 ± 0.17	43.55 ± 0.44
Monoenoic	18.29 ± 0.21	18.35 ± 0.10	19.25 ± 0.13	18.74 ± 0.34
n-6 PUFA				
18:2	0.49 ± 0.01	0.44 ± 0.02	0.47 ± 0.01	0.51 ± 0.01
20:3	0.39 ± 0.05	0.28 ± 0.03	0.37 ± 0.01	0.40 ± 0.05
20:4	14.99 ± 0.26	14.84 ± 0.29	15.07 ± 0.18	14.67 ± 0.24
22:4	3.23 ± 0.83	3.90 ± 0.03	2.90 ± 0.08	3.27 ± 0.11
22:5	2.01 ± 0.09	10.19 ± 0.11	1.53 ± 0.07	1.54 ± 0.08
n-3 PUFA				
22:5 Δ19*t*	—	—	—	—
22:5	0.52 ± 0.20	0.15 ± 0.05	0.30 ± 0.07	0.41 ± 0.02
22:6 Δ19*t*	0.51 ± 0.08	0.95 ± 0.11	—	—
22:6	14.18 ± 0.17	4.26 ± 0.16	13.73 ± 0.08	14.24 ± 0.33
Others	3.02 ± 0.69	2.41 ± 0.60	2.66 ± 0.24	2.68 ± 0.12

[a]Values are % of total fatty acids and are means ± SE of three pools. Brain capillaries and microvessels were obtained from pups at weaning and were lyophilized. After extraction of the lipids and methylation, fatty acid methyl esters were analyzed by gas–liquid chromatography on three columns: CP Sil 88, DB 23 and DB Wax. The chromatographic conditions and the procedures for peak identification are given in the Materials and Methods section. See Table 5 for abbreviation.

TABLE 7

Fatty Acid Composition of Retina[a]

Fatty acids	Groups			
	A	B	C	D
Saturated	43.09	42.62	42.86	42.66
Monoenoic	13.62	12.19	12.20	12.10
n-6 PUFA				
18:2	0.98	0.78	0.78	0.94
20:3	0.29	0.25	0.22	0.23
20:4	11.94	13.07	11.89	11.92
22:4	2.02	3.30	1.94	2.22
22:5	1.53	14.23	1.27	1.40
n-3 PUFA				
22:5 Δ19*t*	0.13	0.05	—	—
22:5	0.75	0.30	0.80	0.79
22:6 Δ19*t*	0.64	1.64	—	—
22:6	23.62	9.58	26.82	25.51
Others	1.39	1.99	1.22	2.23

[a]Values are % of total fatty acids and were determined using one pool. Retina was obtained from pups at weaning and lyophilized. After extraction of the lipids and methylation, fatty acid methyl esters were analyzed by gas–liquid chromatography on three columns: CP Sil 88, DB 23 and DB Wax. The chromatographic conditions and the procedures for peak identification are given in the Materials and Methods section. See Table 5 for abbreviation.

Our objective was furthermore to compare the incorporation of the *trans* DHA into brain and into other tissues. The *trans* isomers of 22:6n-3, 22:5n-3 and 20:5n-3 had been detected in various tissues, such as liver, kidney, heart, adrenals and testes (9,10,30), and the liver was chosen for the comparison with brain structures in this experiment. For the liver of the same weanling animals, 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t* (Table 10) represented only 4.5% of total 22:6n-3, which is similar to the results obtained for the brain structures. In the α-linolenic acid-deficient group, the percentage of *trans* 22:6 ranged from 14.6 to 24.6% in the brain structures. These values are in the same range as those for the liver (25%). It appears that the brain structures are only slightly more protected than other tissues against the incorporation of *trans* isomers. It would be interesting to determine whether this is valid only during brain development or also later in life such as during growth and in maturity.

In this, as well as in previous studies (10,30), the *trans* n-3 PUFA were present only in tissues or lipid classes in which their corresponding *cis* compounds were found. For example, in our experiment, no 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* was

TABLE 8

Fatty Acid Composition of Myelin[a]

Fatty acids	Groups			
	A	B	C	D
Saturated	40.56 ± 0.59	41.34 ± 0.15	41.07 ± 0.85	39.69 ± 0.16
Monoenoic	36.69 ± 0.32	36.20 ± 0.61	36.24 ± 0.55	35.78 ± 0.07
n-6 PUFA				
18:2	0.85 ± 0.03	0.75 ± 0.01	0.78 ± 0.05	0.76 ± 0.02
20:3	1.35 ± 0.17	1.09 ± 0.07	1.26 ± 0.06	1.29 ± 0.06
20:4	8.64 ± 0.43	8.21 ± 0.17	8.81 ± 0.24	9.06 ± 0.21
22:4	3.49 ± 0.18	4.06 ± 0.26	3.78 ± 0.26	4.44 ± 0.31
22:5	0.61 ± 0.14	2.95 ± 0.07	0.81 ± 0.04	0.83 ± 0.06
n-3 PUFA				
22:5 Δ19*t*	0.05 ± 0.01	trace[b]	—	—
22:5	0.19 ± 0.04	0.07 ± 0.03	0.27 ± 0.05	0.33 ± 0.13
22:6 Δ19*t*	0.12 ± 0.02	0.22 ± 0.03	—	—
22:6	3.44 ± 0.34	1.00 ± 0.25	4.07 ± 0.22	4.92 ± 0.48
Others	4.04 ± 0.76	4.10 ± 0.26	2.90 ± 0.30	2.90 ± 0.21

[a]Values are % of total fatty acids and are means ± SE of three pools. Myelin was obtained from pups at weaning and was lyophilized. After extraction of the lipids and methylation, fatty acid methyl esters were analyzed by gas–liquid chromatography on three columns: CP Sil 88, DB 23 and DB Wax. The chromatographic conditions and the procedures for peak identification are given in the Materials and Methods section. See Table 5 for abbreviation.

[b]Trace = <0.01.

TABLE 9

Fatty Acid Composition of Sciatic Nerve[a]

Fatty acids	Groups			
	A	B	C	D
Saturated	46.62 ± 1.43	47.08 ± 0.65	46.44 ± 2.06	46.47 ± 0.62
Monoenoic	37.17 ± 0.43	35.13 ± 0.68	37.52 ± 1.39	37.50 ± 0.35
n-6 PUFA				
18:2	3.67 ± 1.46	2.27 ± 0.29	3.82 ± 0.57	3.73 ± 0.46
18:3	0.06 ± 0.01	0.09 ± 0.05	0.05 ± 0.01	0.05 ± 0.01
20:3	0.72 ± 0.18	0.89 ± 0.07	0.75 ± 0.09	0.76 ± 0.13
20:4	4.66 ± 0.73	6.38 ± 0.36	4.73 ± 0.47	4.82 ± 0.37
22:4	1.01 ± 0.20	2.17 ± 0.28	0.98 ± 0.03	0.96 ± 0.06
22:5	0.31 ± 0.06	1.71 ± 0.21	0.36 ± 0.05	0.34 ± 0.05
n-3 PUFA				
18:3	0.32 ± 0.05	0.07 ± 0.01	0.49 ± 0.10	0.48 ± 0.07
20:5 Δ17*t*	0.06 ± 0.01	0.06 ± 0.01	—	—
20:5	0.16 ± 0.03	—	0.15 ± 0.02	0.15 ± 0.02
22:5 Δ19*t*	0.08 ± 0.24	0.04 ± 0.01	—	—
22:5	0.59 ± 0.14	0.29 ± 0.07	0.51 ± 0.03	0.54 ± 0.05
22:6 Δ19*t*	0.08 ± 0.04	0.28 ± 0.08	—	—
22:6	2.07 ± 0.27	0.86 ± 0.06	2.17 ± 0.32	2.14 ± 0.16
Others	2.41 ± 0.39	2.68 ± 0.32	2.04 ± 0.22	2.04 ± 0.19

[a]Values are % of total fatty acids and are means ± SE of three pools. Sciatic nerve was obtained from pups at weaning and was lyophilized. After extraction of the lipids and methylation, fatty acid methyl esters were analyzed by gas–liquid chromatography on three columns: CP Sil 88, DB 23 and DB Wax. The chromatographic conditions and the procedures for peak identification are given in the Materials and Methods section. See Table 5 for abbreviation.

observed in synaptosomes, brain microvessels, retina and myelin; in these cases also no *all cis* EPA was observed. Sciatic nerve contains only some *trans* EPA and only small quantities of EPA. The metabolism of *trans* n-3 PUFA thus appears to parallel the metabolism of *all cis* n-3 PUFA. When *all cis* α-linolenic acid is deficient, *trans* PUFA are incorporated to a greater extent into the brain structures. However, their levels never reach the levels of the corresponding *cis* compounds in rats that are not deficient in α-linolenic acid. It is possible that the level of 18:3

TABLE 10

Content of *trans* 22:6n-3 in Various Tissues[a]

	Groups	
	A	B
Synaptosomes	3.0	21.2
Brain microvessels	3.5	18.2
Retina	2.6	14.6
Myelin	3.4	18.0
Sciatic nerve	3.7	24.6
Liver	4.5	25.0

[a]Values are % of total 22:6.

Δ9*c*,12*c*,15*t* (about 600 mg/kg diet) was not sufficient compared to the initial level of linolenic acid (about 2500 mg/kg diet). However, as in previous experiments, it appears that the *cis* n-3 PUFA were probably more easily desaturated and elongated than the corresponding *trans* compounds. This is consistent with the levels of *trans* long-chain n-3 PUFA incorporated into these tissues compared with the percentage of their precursor in the diet (Table 3).

We know that geometrical isomers of α-linolenic acid can be present in the human diet. The low-calorie spreads marketed in France can contain up to 28% of their 18:3 as *trans* compounds (5). Moreover, in some frying oils about 40% of linolenic acid was of *trans* configuration (4). It can therefore be argued that the ratios of *trans* isomers of α-linolenic acid used in the present experiments (47.5 of total 18:3 in group A) were quite realistic. The compounds can be desaturated and elongated *in vivo* and thus provide *trans* isomers of EPA or DHA, even in brain structures. Although our studies were done in rats, evidence for the occurrence of isomers of EPA and DHA in human tissues was brought forward recently by Chardigny *et al.* (31) who identified the *trans* isomer of EPA in most of the fourteen samples of human platelets studied. The *trans* isomer of DHA was also detected but to a lesser extent and only in four samples. It will be necessary to determine whether or not these amounts could have physiological implications. In another study done on human platelets (32), 20:5 Δ5*c*,8*c*,11*c*,14*c*,17*t* was shown to be a less powerful antiaggregant than EPA, and 22:6 Δ4*c*,7*c*,10*c*,13*c*,16*c*,19*t* was shown to be more efficient than DHA in inhibiting aggregation induced by arachidonic acid.

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Hepatic Phospholipid Molecular Species in the Guinea Pig. Adaptations to Pregnancy

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Incorporation of polyunsaturated fatty acids (PUFA), particularly 22:6n-3, into fetal brain at specific gestational ages is critical for development of normal brain function. We have studied adaptations to maternal liver phospholipid molecular species compositions that may be related to the supply of PUFA to fetal brain. The increment of 22:6n-3 in brain phosphatidylethanolamine (PE) was maximal at day 25 to day 35 of gestation, consistent with early prenatal development of guinea pig brain. At the same gestational ages, there was a transient increase in maternal liver concentration of 16:0/22:6 phosphatidylcholine (PC), which preceded the progressive increase in total PC concentration toward term (day 68). This effect was specific for the *sn*-1 16:0 species, as there was no significant increase in 18:0/22:6 PC concentration. These results are consistent with a specific role for 16:0/22:6 PC in the directed supply of 22:6n-3 from maternal liver to the fetus. Concentrations of all PE species in maternal liver decreased at day 25 and day 35 of gestation. The gradual accumulation of 22:6n-3 in fetal liver throughout gestation did not correlate with the pattern of acquisition of 22:6n-3 into fetal brain PE. Maternal plasma PC and cholesterol concentrations decreased dramatically by day 25 of gestation, and remained low until term. This hypolipidemia of pregnancy in the guinea pig may be due to increased lipase-mediated turnover of plasma lipoproteins and contrasts strongly with the well-characterized hyperlipidemia in human and rat gestation.

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Optimal fetal growth requires an adequate supply of essential polyunsaturated fatty acids (PUFA) at defined periods in gestation. Failure to acquire sufficient and appropriate PUFA at such critical periods in fetal development may have irreversible and harmful consequences for post-natal growth and function. The major PUFA arachidonate (20:4n-6) and docosahexaenoate (22:6n-3) must be derived from the essential fatty acids linoleate (18:2n-6) and α-linolenate (18:3n-3) in the maternal diet. Studies in preterm human infants fed formula either lacking or supplemented with marine oils (1,2) have shown that the capacity of the fetus and neonate to desaturate and chain elongate 18:2n-6 and 18:3n-3 is inadequate to meet the demands of rapid growth in development. The conclusion from these and many other studies is that the bulk of the long-chain PUFA must be synthesized by the mother and supplied to the fetus and neonate either by placental transport or through the milk. Further evidence to support this view was provided by analysis of phospholipid fatty acid composition in cerebral cortex obtained from victims of Sudden Infant Death Syndrome, who had been born at term (3). Brain phosphatidylethanolamine (PE) 22:6n-3 was deficient in infants who had been fed formula not supplemented with preformed 22:6n-3 as compared with breast-fed infants. The mechanisms regulating the maternal synthesis of 20:4n-6 and 22:6n-3 destined for supply to the fetus are still not understood. Indeed, despite knowledge of the maternal hyperlipidemia of human pregnancy for almost 150 years (4), it is still not clear which lipid or lipoprotein subfraction in the circulation is the principal carrier of PUFA to the placenta.

A significant proportion of the 22:6n-3 delivered to the fetus is destined for incorporation into neuronal membranes of the developing brain, preferentially as PE (5). The period of 22:6n-3 accumulation in brain PE is associated with the process of neurite extension and axonal formation and precedes the brain growth spurt characterized by the incorporation of saturated fatty acids into brain myelin (6). The timing of maximal 22:6n-3 incorporation into brain PE and brain development differs considerably between animal species (7). Consequently, for any given species, any adaptations of maternal lipid metabolism proposed to be involved in the directed supply of 22:6n-3 must correlate temporally with its accumulation in fetal brain.

In human development, 22:6n-3 incorporation into fetal brain is initiated early in gestation, at about 16 wk (8,9), and continues into early post-natal life. This process in the rat is restricted to the post-natal period (10). Analyses of the ontogeny of the accumulation of 22:6n-3 in fetal guinea pig brain phospholipids have not previously been reported. However, neuronal differentiation is essentially prenatal in the guinea pig (11), and the timing of neuritogenesis at about day 40 of gestation term (term = 68 d) (12) suggests that brain 22:6n-3 accumulation should also be substantially prenatal.

In maternal rat liver (13) and in maternal human plasma (14), the period of 22:6n-3 accumulation by perinatal brain is preceded by an increased concentration of the phosphatidylcholine (PC) molecular species, 16:0/22:6 PC. This suggested that 16:0/22:6 might be a preferential carrier for transport of 22:6n-3 from the maternal liver either to fat stores destined for use during lactation or to the placenta for supply to the developing fetus.

In the present study we have characterized the molecular species compositions of PC and PE in fetal and maternal liver and in maternal plasma throughout guinea pig gestation, and have compared the adaptations with the ontogeny of PUFA accumulation in fetal brain.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; FA, fatty acid(s); GC, gas chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid(s); TFE, trifluoroethanol; UV, ultraviolet.

MATERIALS AND METHODS

Materials. HPLC grade methanol and trifluoroethanol (TFE) were obtained from Rathburn Ltd. (Walkerburn, Scotland). Chloroform, choline chloride and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Merck Ltd. (Poole, Dorset, United Kingdom). All other reagents and lipid standards were from Sigma (Poole, Dorset, United Kingdom).

Animal procedures. Outbred adult female Dunkin–Hartley guinea pigs were fed maintenance diet (FD1; Special Diet Services, Witham, Essex, United Kingdom) *ad libitum* before mating and throughout pregnancy. The diet contained 3.4% fat by weight, and its fatty acid (FA) content, expressed relative to total esterified fatty acids, was 22.2% saturated FA, 30.9% monounsaturated FA, 22.7% linoleic acid (18:2n-6), 16.3% α-linolenic acid (18:3n-3) and 7.9% PUFA, mainly 20:4n-6. At gestational ages 25, 35, 55 and 68 (term) days, fetal guinea pigs were delivered by Caesarian section (15). Maternal livers, fetal livers and fetal brains were collected, immediately frozen in liquid nitrogen and stored at –20°C. Obtaining fetal tissues at earlier gestational ages was not technically feasible.

HPLC analysis of phospholipid molecular species. Liver or brain tissue, approximately 100 mg, was homogenized in 0.8 mL of 0.9% (wt/vol) NaCl with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany). One hundred nmoles each of 14:0/14:0 PC in TFE and 14:0/14:0 PE in chloroform were added to liver homogenates as internal standards. Lipids were extracted with chloroform/methanol (16); PC and PE were purified using aminopropyl BondElut cartridges (Jones Chromatography, Hengoed, Mid Glamorgan, United Kingdom) (15,17). Intact liver PC and PE molecular species were resolved by reverse-phase HPLC on a 25 cm × 4.6 mm APEX II ODS column (Jones Chromatography) (15,18) maintained at 50°C using a mobile phase of methanol/water (925:75, vol/vol) containing 40 mM choline chloride. These isocratic conditions were chosen as a compromise between optimal resolution of phospholipid species containing PUFA, and the ability to resolve 16:0/16:0 and 16:0/18:1 species effectively. Although adding acetonitrile up to 20% (by vol) improved the resolution of PUFA-containing species, this modification resulted in co-elution of 16:0/18:1 and 18:0/22:6 species, and consequently was not used. The elevated temperature of 50°C was essential to control the elution order of phospholipid species (18). The mass of eluted PC and PE molecular species was determined by fluorescence detection following post-column derivatization with DPH (18). Ultraviolet (UV) absorbance at 205 nm, recorded simultaneously with the fluorescence signal, was routinely used to confirm the identity of molecular species. For all chromatographic peaks of PC and PE, the principal component species, determined by gas chromatography (GC), is quoted. Calculation of the ratio of UV absorbance to fluorescence responses for each eluted peak at each gestational age confirmed that the gestational changes observed in phospholipid composition were substantially due to the major species indicated.

Analysis of brain phospholipid fatty acids by GC. Brain PC and PE fractions eluted from BondElut cartridges were dried under nitrogen and dissolved in 20 μL of chloroform/methanol (2:1, vol/vol). Twenty nmoles of 17:0/17:0 PC was added as internal standard. Fatty acid methyl esters, prepared by transmethylation of phospholipids with sodium methoxide, were resolved by GC using a fused silica capillary column (30 m × 0.25 mm, DB225; Jones Chromatography).

Statistical analysis. Statistical analysis was carried out using the Mann–Whitney U-test.

RESULTS

Fatty acid composition of fetal guinea pig brain PC and PE. The fatty acid composition of the major phospholipid classes PC and PE in fetal guinea pig brain were analyzed at intervals throughout gestation to define the timescale for the acquisition of the PUFAs 22:6n-3 and 20:4n-6. The concentration (mole %) of the principal FAs in fetal brain PC, 16:0 and 18:1n-9, remained essentially constant between day 25 and day 68 (term) (Fig. 1). The corresponding values for 20:4n-6 and 22:6n-3 both increased significantly in early gestation, reaching maxima at day 55. These FAs, however, remained minor components of fetal brain PC throughout gestation, and neither contributed more than 6% to total PC. In contrast, the gestational changes in fetal brain PE were considerably more marked. Both 20:4n-6 and 22:6n-3 were major components of brain PE at term (Fig. 2); the concentration (mole %) of 22:6n-3 in brain PE increased dramatically between gestational ages of day 25 and day 35, and then continued to increase steadily toward term. The accumulation of 20:4n-6 in brain PE occurred slightly earlier in gestation. At day 25, the earliest time

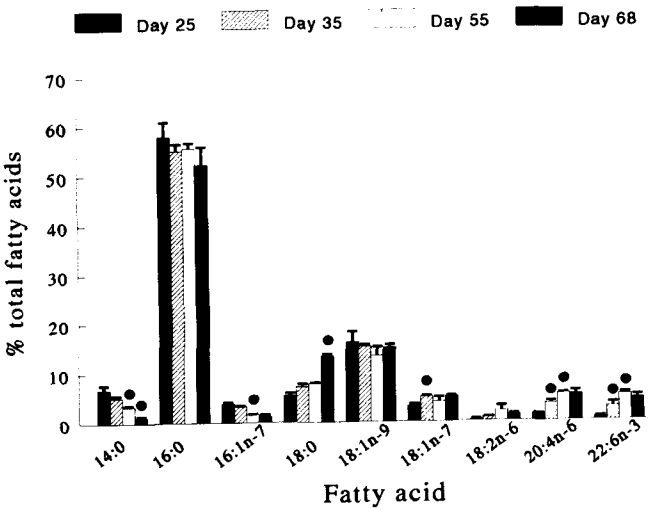

FIG. 1. Phosphatidylcholine (PC) fatty acid composition (mole %) of developing fetal guinea pig brain during gestation. Concentrations of PC fatty acids were measured by gas chromatography. The fatty acids reported account for > 97% of the total fatty acids present. Values are the mean ± SD of n = 6 samples at each gestational age. Circles indicate values that were significantly different ($P > 0.05$) from the preceding gestational age.

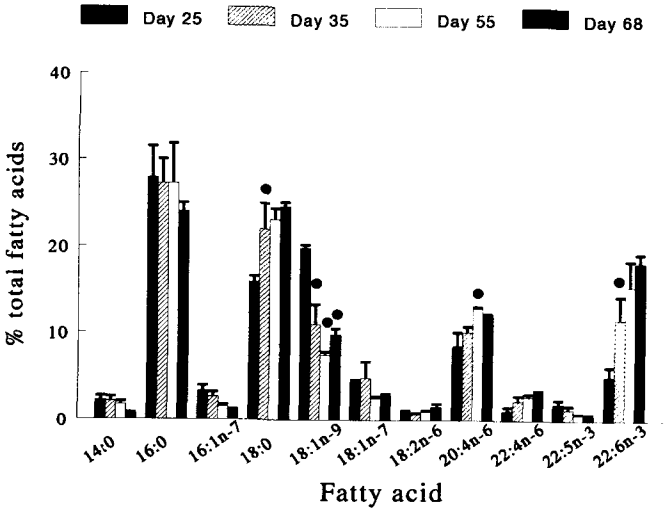

FIG. 2. Phosphatidylethanolamine (PE) fatty acid composition (mole %) of developing fetal brain during gestation. The concentrations of PE fatty acids were measured by gas chromatography. Fatty acids reported accounted for >97% of the total fatty acids detected. Values are the mean ± SD of n = 6 samples at each gestational age. Circles indicate values that were significantly different ($P > 0.05$) from the preceding gestational age.

point feasible for analysis, brain PE was already enriched in 20:4n-6 to a value of 80% of that measured at term (Fig. 2). Over the same time period in early gestation, the increases in PUFA components in brain PE were accompanied by a parallel increase in 18:0 and a decrease in 18:1n-9 (Fig. 2).

Molecular species composition of maternal guinea pig liver PC. Ten PC molecular species were consistently identified in both maternal and fetal guinea pig liver, and these species accounted for more than 97% of total PC (15). Total PC concentration in maternal liver increased significantly over the second half of gestation, from a nonpregnant value of 8.59 ± 1.91 to 14.10 ± 0.41 μmol/g wet weight at term (Table 1). Within this overall increase, there were strikingly different temporal patterns for changes to the concentrations of individual molecular species. For species containing long-chain PUFA, significantly increased concentrations were evident only in those with 16:0 at the *sn*-1 position, and these were seen early in gestation. For example, the concentration of 16:0/22:6 PC increased to a maximum at day 35 of gestation, and then decreased toward term (Table 1). In contrast, no significant gestational change was observed for the concentration of 18:0/22:6 PC. Similarly, the concentration of 16:0/20:4 PC increased threefold to a maximum at the earliest time measured (day 25) and then decreased until term, whereas no significant alteration was observed for 18:0/20:4 PC (Table 1). The patterns of changes to concentrations of PC species containing 18:2n-6 during gestation were completely different from those observed in PUFA-containing PC species. Neither 16:0/18:2 PC nor 18:0/18:2 PC concentration changed significantly until day 35 of gestation, after which the concentrations of both species continued to increase until term. In addition to these alterations to hepatic concentrations of polyunsaturated PC species in the pregnant guinea pig, significant changes were apparent in the concentrations of both disaturated and monounsaturated PC species. The concentration of 16:0/16:0 PC increased gradually throughout gestation, whereas that of 18:0/18:1 PC reached a maximum at day 35 of gestation and then decreased to nonpregnant values by term.

Molecular species composition of maternal guinea pig liver PE. In contrast to the gestational increase in maternal liver PC concentration, the total PE concentration decreased in early gestation from a nonpregnant value of 9.63 ± 0.21 to 6.19 ± 0.79 μmol/g wet weight at

TABLE 1

Maternal Guinea Pig Liver Phosphatidylcholine Composition[a]

	Phosphatidylcholine (nmol/g)				
	Gestational age (d)				
Molecular species	Nonpregnant	25	35	55	68
16:0/18:3	279 ± 90	268 ± 84	160 ± 4[b,c]	299 ± 164	158 ± 86
16:0/22:6	902 ± 106	1458 ± 309[b]	1614 ± 287[b]	1003 ± 128[c,d]	795 ± 298[c,d]
16:0/20:4	144 ± 29	462 ± 152[b]	397 ± 96[b]	331 ± 140[b]	204 ± 91[c,e]
16:0/18:2	1880 ± 376	2437 ± 310	2876 ± 624[b]	3295 ± 91[b,c]	3298 ± 268[b,c]
16:0/16:0	224 ± 66	471 ± 157[b]	609 ± 253	744 ± 201[b,c]	607 ± 127[b]
16:0/18:1	630 ± 93	828 ± 183	1286 ± 466[b]	686 ± 20[d]	766 ± 53[d,e]
18:0/22:6	1064 ± 386	1274 ± 227	1297 ± 295	1043 ± 190	1415 ± 283
18:0/20:4	609 ± 283	1022 ± 274	960 ± 147	669 ± 95	583 ± 88
18:0/18:2	2188 ± 1417	2534 ± 238	4702 ± 1892[c]	5265 ± 759[b,c]	5686 ± 700[b,c]
16:0/18:0	584 ± 91	104 ± 10[b]	437 ± 71[c]	395 ± 95[b,c]	583 ± 111[c,e]
Total	8592 ± 1913	11686 ± 1592	14720 ± 4048[b]	13971 ± 452[b]	14096 ± 410[b,c]

[a]Results of high-performance liquid chromatography analyses of phosphatidylcholine molecular species compositions in maternal guinea pig liver expressed as mean ± SD; n = 3 samples/gestational age.

[b–e]Results significantly ($P < 0.05$) different from nonpregnant, day 25-pregnant, day 35-pregnant and day 55-pregnant, respectively. Maternal liver mass (gram wet weight): Nonpregnant, 21.1 ± 2.4; day 25, 40.7 ± 1.4; day 35, 34.0 ± 3.9; day 55, 31.1 ± 2.1; day 68, 29.4 ± 4.6.

TABLE 2

Maternal Guinea Pig Liver Phosphatidylethanolamine Composition[a]

	Phosphatidylethanolamine (nmol/g)				
	Gestational age (d)				
Molecular species	Nonpregnant	25	35	55	68
16:0/22:6	1485 ± 401	1039 ± 153	1107 ± 106	1573 ± 28^{c,d}	1503 ± 115^{c,d}
16:0/20:4	512 ± 127	116 ± 14^{b}	625 ± 81^{c}	620 ± 94^{c}	597 ± 178^{c}
16:0/18:2	358 ± 130	615 ± 142	475 ± 99	679 ± 79^{b,d}	684 ± 30^{b,d}
16:0/16:0	573 ± 207	148 ± 44^{b}	305 ± 80^{b,c}	525 ± 54^{c,d}	642 ± 37^{c-e}
18:0/22:6	2890 ± 721	1966 ± 502	1541 ± 40^{b}	3070 ± 457^{c,d}	3507 ± 140^{c-e}
18:0/20:4	1784 ± 368	1105 ± 298^{b}	1248 ± 47^{b}	1432 ± 10^{b-d}	1264 ± 42^{b,c}
18:0/18:2	1965 ± 285	1198 ± 293^{b}	1162 ± 71^{b}	1516 ± 365^{c,d}	2701 ± 223^{b-e}
Total	9628 ± 2127	6188 ± 795^{b}	6311 ± 150^{b}	9414 ± 927^{c,d}	10879 ± 319^{c-e}

[a]Results of high-performance liquid chromatography analyses of phosphatidylethanolamine molecular species compositions in maternal guinea pig liver expressed as mean ± SD; n = 3 samples/gestational age.

[b–e]Results significantly ($P < 0.05$) different from nonpregnant, day 25-pregnant, day 35-pregnant and day 55-pregnant, respectively.

day 25 of gestation (Table 2), and then subsequently returned to the original concentration by term. Moreover, there was no significant temporal discrimination in concentration changes between the individual molecular species of PE. The concentrations of all the major PE species in maternal liver tended to decrease early in gestation and then increase again toward term. Again, in contrast to PC, the major changes to maternal liver PE were apparent in species containing *sn*-1 18:0 rather than *sn*-1 16:0 (Table 2).

Lipid composition of maternal guinea pig plasma. Concentrations of total cholesterol and PC decreased significantly in maternal plasma by day 25 of gestation compared with the nonpregnant values (Table 3), and both then remained low throughout the remainder of gestation. Plasma triglyceride concentration did not change significantly at any time in gestation. Molecular species analysis of plasma PC showed that all species containing 22:6n-3, 20:4n-6 and 18:2n-6 decreased to similar extents in gestation (Table 3).

Molecular species composition of fetal guinea pig liver PC and PE. The apparent increase in fetal liver total PC between day 35 and day 68 of gestation was not significant. In contrast, fetal liver PE concentration doubled over the same period (Table 4). The mass of fetal liver at day 25 was too low (10 ± 2 mg) for molecular species analysis. Molecular species of PC and PE containing 22:6n-3 and 20:4n-6 were already substantially enriched in fetal liver at day 35 of gestation. The concentrations of all PC and PE species containing 22:6n-3 then increased

TABLE 3

Maternal Guinea Pig Plasma Lipid Composition[a]

	Gestational age (d)				
	Nonpregnant	25	35	40	68
Cholesterol (mmol/L)	0.9 ± 0	0.4 ± 2^{b}	0.7 ± 0.4	0.5 ± 0.1^{b}	0.2 ± 0.1^{b}
Triglyceride (mmol/L)	0.60 ± 0.02	0.52 ± 0.13	0.90 ± 0.59	0.68 ± 0.13	0.47 ± 0.10
Phosphatidylcholine (mmol/L)	0.216 ± 0.007	0.081 ± 0.004^{b}	0.061 ± 0.073^{b,c}	0.073 ± 0.009^{b}	0.086 ± 0.017^{b}
	Phosphatidylcholine (nmol/mL)				
	Gestational age (d)				
Molecular species	Nonpregnant	25	35	40	68
16:0/22:6	12.3 ± 0.8	2.8 ± 0.5^{b}	1.9 ± 0.2^{b,c}	1.9 ± 0.6^{b}	2.7 ± 0.5^{b,d}
16:0/20:4	3.4 ± 0.7	2.6 ± 0.4^{b}	1.0 ± 0.1^{b,c}	1.3 ± 0.3^{b-d}	0^{b-e}
16:0/18:2	43.7 ± 4.3	19.7 ± 4.8^{b}	14.7 ± 2.3^{b}	14.9 ± 0.7^{b}	19.4 ± 3.7^{b}
16:0/18:1	17.5 ± 1.2	4.1 ± 1.2^{b}	5.3 ± 3.2^{b}	2.6 ± 0.7^{b}	3.5 ± 1.3^{b}
18:0/22:6	24.4 ± 0.5	1.4 ± 0^{b}	1.8 ± 1.0^{b}	2.8 ± 0.3^{b,c}	3.2 ± 1.0^{b,c}
18:0/20:4	14.3 ± 5.5	0.4 ± 0.1^{b}	0.5 ± 0.1^{b}	1.9 ± 0.4^{b-d}	2.1 ± 0.1^{b-d}
18:0/18:2	103.2 ± 3.2	50.1 ± 6.0^{b}	28.3 ± 1.5^{b,c}	44.8 ± 5.6^{b,d}	54.8 ± 11.7^{b,d}

[a]Results of analyses of cholesterol, triglyceride and phosphatidylcholine concentrations, and high-performance liquid chromatography analysis of phosphatidylcholine species composition in maternal guinea pig plasma expressed as mean ± SD; n = 3 samples/gestational age.

[b–e]Results significantly ($P < 0.05$) different from nonpregnant, day 25-pregnant, day 35-pregnant and day 55-pregnant, respectively.

TABLE 4

Fetal Guinea Pig Liver Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) Molecular Species Composition[a]

	Phosphatidylcholine and phosphatidylethanolamine (nmol/g)		
	Gestational age (d)		
Molecular species	35	55	68
16:0/22:6 PC	421.5 ± 78.2	428.5 ± 60.9	639.2 ± 20.8[b]
16:0/20:4 PC	213.7 ± 91.0	150.4 ± 31.2	188.5 ± 25.6
18:0/22:6 PC	223.6 ± 48.5	391.0 ± 62.3	466.2 ± 15.3[b]
18:0/20:4 PC	110.0 ± 34.6	205.7 ± 70.5	151.2 ± 32.7
Total PC	5352 ± 1360	7198 ± 1087	7875 ± 928
16:0/22:6 PE	371.4 ± 30.0	1061.2 ± 18.8[b]	941.3 ± 39.9
16:0/20:4 PE	151.8 ± 28.9	111.6 ± 14.2	108.3 ± 32.6
18:0/22:6 PE	737.2 ± 49.0	1153.9 ± 30.7[b]	1302.5 ± 107.8[b]
18:0/29:4 PE	758.2 ± 74.9	627.5 ± 21.3	760.4 ± 11.2[b]
Total PE	2880 ± 470	4845 ± 523	7057 ± 1544[b]

[a]Results of high-performance liquid chromatography analysis of developing fetal guinea pig liver PC and PE expressed as mean ± SD nmol/g wet tissue; n = 6 samples/gestational age.

[b]Values that differed significantly ($P < 0.05$) from the preceding gestational age. The wet weights of fetal livers were 0.29 ± 0.08 g (day 35), 2.79 ± 0.18 g (day 55) and 5.94 ± 0.83 g (day 68, term).

gradually until term. By contrast, concentrations of PC and PE species containing 20:4n-6 did not significantly change between day 35 and day 68 of gestation.

DISCUSSION

The results from this study showed, for the first time, the patterns of changes of maternal and fetal guinea pig liver PC and PE molecular species and their temporal relationship to the accumulation of 22:6n-3 in fetal guinea pig brain PE. Differentiation and functional maturation of the guinea pig brain are essentially prenatal events (4,11,12). Our results confirm that, as might be expected, the accumulation of 22:6-3 in fetal brain PE was also substantially prenatal. The period of most rapid accumulation was between day 25 and day 35 of gestation (Fig. 2), which preceded both the reported onset of electrical activity (days 46–48) (19) and the expression of the neurite growth cone-associated protein GAP-43 (day 40) (20). This result is consistent with the incorporation of 22:6n-3 into the membranes of maturing neurones prior to, and in support of, neurite extension and axon formation. One consequence of this demonstration of the early timescale for the accumulation of 22:6n-3 in fetal guinea pig brain is that any adaptations to maternal lipid metabolism that might regulate the supply of 22:6n-3 to the developing fetus must also be functional early in gestation. Analysis of hepatic PC composition of the pregnant guinea pig, compared with control values in nonpregnant animals, demonstrated specific increased concentrations of 16:0/22:6 PC and 16:0/20:4 PC at day 25 and day 35 of gestation that were not apparent in 18:0/22:6 PC, 18:0/20:4 PC or in PC species containing *sn*-2 18:2. These observations are consistent with comparable measurements in human and rat pregnancy. The concentration of *sn*-1 16:0 PC species, including 16:0/22:6 PC, increased in the plasma of pregnant women in late gestation, with no change in the concentrations of *sn*-1 18:0 PC species (14). Similarly, there was a dramatic increase in 16:0/22:6 PC concentration in maternal rat liver and plasma at term (13) in anticipation of the lactational supply of 22:6n-3 to the rat neonate (10).

Comparison between these three animal species suggests a possible role for 16:0/22:6 PC in the directed transport of 22:6n-3 from the maternal liver to the fetus or neonate. Previous reports of the analysis of the specificity of PC synthesis in adult rat hepatocytes (21) and fetal guinea pig liver (15) further suggest a number of potential regulatory mechanisms. For both the rat and guinea pig, there is preferential synthesis *de novo via* CDPcholine of *sn*-1 16:0 PC species; *sn*-1 18:0 PC species are formed largely by acyl remodeling and exhibit longer turnover times. Consequently, assuming no alteration to the mechanisms of PC acyl remodeling, an increased rate of total hepatic PC synthesis might be expected to result in a shift from *sn*-1 18:0 to *sn*-1 16:0 PC species. Any contribution of altered fatty acid supply to these mechanisms is unclear, as the composition of the diacylglycerol substrate pool for hepatic PC synthesis is not known. Finally, a significant portion of the 22:6n-3, and essentially all the 20:4n-6 in guinea pig liver is derived by *N*-methylation of the entire liver PE pool (15). As liver PE is substantially enriched in species containing 22:6n-3 and 20:4n-6 as compared with PC, increased flux through *N*-methylation would lead to an increased concentration of these FAs in the product PC. It is possible that the decreased maternal liver PE concentration at days 25 and 35 of gestation (Table 2), coincident with the increased PC concentration at the same gestational ages, is a reflection of such an increased rate of conversion by the *N*-methylation pathway.

The analysis of plasma lipid concentrations in the pregnant guinea pig (Table 3) demonstrates clearly that the regulatory mechanisms responsible for supply of 22:6n-3 to the developing fetus cannot be completely identical for all animal species. In contrast to the hyperlipidemia of late gestation in pregnant women (4,22) and the pregnant rat (23), the pregnant guinea pig exhibited a pronounced hypolipidemia from early gestation until term. Previous reports have shown that the guinea pig has a unique lipoprotein composition, with very low concentrations of high density lipoprotein in adult plasma, and that concentrations of cholesterol and triglyceride are greater in fetal than in maternal guinea pig plasma at term (24). In addition, circulating triglyceride has a half-life of minutes in the guinea pig (25), placental hydrolysis of esterified lipid is rapid (26,27), and circulating maternal plasma lipids contribute significantly to the massive hepatic and plasma hyperlipidemia of the fetal guinea pig in late gestation (24). This evidence suggests that the rapid rate of lipoprotein clearance in the guinea pig, enhanced in pregnancy by the high activity of placental lipoprotein lipase (26), is a major determinant of circulating lipid concentration. Consequently, under such conditions, the rate of maternal hepatic lipoprotein synthesis and secretion may not correlate with the plasma lipid concentration. In effect, the increased contents of 20:4n-6 and 22:6n-3 in maternal liver, derived either directly from the diet or by endogenous synthesis from dietary essential fatty acids, may act as transient store before rapid mobilization, hepatic lipoprotein secretion and placental transfer to the fetus.

The pattern of change of PC and PE molecular species containing 22:6n-3 in fetal guinea pig liver between day 35 and day 68 of gestation (Table 4) did not correlate with the timing of acquisition of 22:6n-3 in fetal brain PE. The rapid growth of the fetal liver after day 35 (Table 4) suggests only a limited possible role for this organ in the regulation of the 22:6n-3 supply to fetal brain early in gestation. Additionally, the fetal guinea pig liver in early gestation is immature and contains relatively few hepatocytes (27), further restricting any potential for involvement in essential fatty acid supply to other tissues.

While the coordination between increased maternal liver 16:0/22:6 PC and the accumulation of 22:6n-3 in fetal brain PE suggests a specific adaptation of maternal liver phospholipid metabolism to supply the requirements of the developing fetus, confirmation of this hypothesis requires extensive metabolic analysis defining rates of synthesis, turnover and catabolism of individual molecular species in the various phospholipid pools. Such information will be essential to determine, for instance, whether plasma lipid concentrations decrease in the pregnant guinea pig as a consequence of increased lipoprotein clearance, and whether these decreased concentrations are consistent with increased flux of lipid through the plasma lipoprotein pools. Similarly, while the absence of any alteration to the composition of PUFA-containing phospholipids in fetal liver coincident with the accumulation of 22:6n-3 in fetal brain PE at day 25 and day 35 of gestation suggests little involvement of fetal liver metabolism in directing PUFA supply to the fetal brain, this supposition needs to be validated by metabolic analysis. Such studies are currently being undertaken in our laboratory.

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Dietary Protein Deficiency Affects n-3 and n-6 Polyunsaturated Fatty Acids Hepatic Storage and Very Low Density Lipoprotein Transport in Rats on Different Diets

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Fatty livers and the similarity between the skin lesions in kwashiorkor and those described in experimental essential fatty acid (EFA) deficiency have led to the hypothesis that protein and EFA deficiencies may both occur in chronic malnutrition. The relationship between serum very low density lipoprotein (VLDL) and hepatic lipid composition was studied after 28 d of protein depletion to determine the interactions between dietary protein levels and EFA availability. Rats were fed purified diets containing 20 or 2% casein and 5% fat as either soybean oil rich in EFA, or salmon oil rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, or hydrogenated coconut oil poor in EFA. Animals were divided into six groups, SOC (20% casein + 5% soybean oil), SOd (2% casein + 5% soybean oil), COC (20% casein + 5% hydrogenated coconut oil), COd (2% casein + 5% hydrogenated coconut oil), SAC (20% casein + 5% salmon oil) and SAd (2% casein + 5% salmon oil). After 28 d, liver steatosis and reduced VLDL-phospholipid contents ($P < 0.001$) were observed in protein-deficient rats. In protein deficiency, triacylglycerol and phospholipid fatty acid compositions in both liver and VLDL showed a decreased polyunsaturated-to-saturated fatty acid ratio. This ratio was higher with the salmon oil diets and lower with the hydrogenated coconut oil diets. Furthermore, independent of the oil in the diet, protein deficiency decreased linoleic and arachidonic acids in VLDL phospholipids. Conversely, despite decreased proportions of EPA at low protein levels, DHA levels remained higher in rats fed salmon oil diets. While in rats fed the hydrogenated coconut oil-fed diets the amount of 22:5n-6 was lower in liver, it was higher in VLDL lipids at low protein levels. Both EPA and arachidonic acid are precursors of eicosanoids and their diminution may be related to certain clinical symptoms seen in infants suffering from kwashiorkor.

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In protein malnutrition, lipoprotein formation and function appear to be altered, leading to liver steatosis (1). The occurrence of fatty liver and the similarity between the skin lesions (the loss of hair, dry scaly dermatitis with a tendency for superinfection and increased water permeability) in kwashiorkor and those seen in experimental essential fatty acids (EFA) deficiency have led to the hypothesis that protein and EFA deficiencies may both occur in chronic malnutrition (2,3). Hill and Holman (4) reported the effects of various dietary protein levels (5 to 40%) on EFA availability in rats fed hydrogenated coconut oil (CO) and noted that EFA deficiency symptoms are amplified at low protein intake.

Food intakes of infants before hospital admission are difficult to determine, and data on polyunsaturated fatty acids (PUFA) composition reported in several studies are inconsistent (5). It was found in some studies on malnourished children that PUFA were lowered in plasma lipids. Yet in other studies, both linoleic and arachidonic acids (3), or arachidonic alone (6), or linoleic acid in kwashiorkor and arachidonic acid in marasmus (7), were found to be decreased in plasma lipids. Thus, the data indicated a relationship between protein levels and the development of EFA deficiency. It is difficult to study these relationships with accuracy because numerous dietary deficiencies are usually associated with kwashiorkor.

Research on protein malnutrition has focused primarily on protein metabolism. Limited attention has been given to the effects of, and the interactions between, protein and different dietary fatty acids. Human EFA metabolism is quite distinct from that of the rat, and the results of the present study should therefore be extrapolated to humans only with great care. Yet, the rat is a good model to study both protein malnutrition (8) and EFA deficiency (9).

In our previous paper (10) we provided evidence for the effects of protein depletion on the pool size and composition of serum very low density lipoprotein (VLDL), and that VLDL composition was modified dependent on the origin and the quantity of the protein in the low protein diets. In the present experiments, fatty acid and protein intakes were monitored with great accuracy throughout the short-term feeding trials (28 d). Our goal was to assess the qualitative and quantitative differences in liver and VLDL fatty acid composition in growing rats fed combinations of adequate or low levels of casein with soybean oil (SO) (adequate in EFA), CO (deficient in EFA) or salmon oil (SA) (rich in long-chain n-3 PUFA). The present study was also undertaken to determine whether a short period of protein deficiency would also diminish n-3 and n-6 PUFA availability independent, or not, of the fat source and whether changes found in VLDL serum levels would be accompanied by similar alterations in apolipoprotein and lipid composition.

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Abbreviations: Apo, apolipoprotein; CO, hydrogenated coconut oil; COC, 20% casein + 5% hydrogenated coconut oil; COd, 2% casein + 5% hydrogenated coconut oil, DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetate (disodium); EFA, essential fatty acids; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PL, phospholipids; PUFA, polyunsaturated fatty acids; SA, salmon oil; SAC, 20% casein + 5% salmon oil; SAd, 2% casein + 5% salmon oil; SFA, saturated fatty acids; SO, soybean oil; SOC, 20% casein + 5% soybean oil; SOd, 2% casein + 5% soybean oil; TG, triacylglycerols; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Animals and diets. Sixty male Wistar rats (Iffa Credo, l'Arbresle, Lyon, France) initially weighing 83 ± 5 g were allowed free access to an adequate diet (20% casein and 5% olive oil) for 10 d. After this adaptation period, when body weights reach 115 ± 7 g, the animals were fed purified diets containing 20 or 2% casein and 5% fat as either SO rich in EFA or SA rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, or CO poor in EFA. Rats were randomly divided into six groups, SOC (20% casein + 5% soybean oil), SOd (2% casein + 5% soybean oil), COC (20% casein + 5% hydrogenated coconut oil), COd (2% casein + 5% hydrogenated coconut oil), SAC (20% casein + 5% salmon oil) and SAd (2% casein + 5% salmon oil). The diets are described in Table 1, and the fatty acid compositions of the various oils are described in Table 2.

The six diets were isocaloric (16.28 MJ/kg) and contained identical amounts of lipids, vitamins, minerals and fiber. Animals were kept in wire-bottom cages at constant temperature (24°C) and humidity (60%) at a 12-h light cycle (0700–1900). The rats ate and drank *ad libitum*. The general guidelines for the care and use of laboratory animals (Council of European Communities, 1986) (11) were followed.

TABLE 1

Diet Compositions[a] (g/kg diet)

	Soybean oil (SO) diets		Coconut oil (CO) diets		Salmon oil (SA) diets	
	SOC	SOd	COC	COd	SAC	SAd
Milk casein[b]	200	20	200	20	200	20
DL-Methionine[c]	3	3	3	3	3	3
Cornstarch[d]	587	767	587	767	587	767
Sucrose	50	50	50	50	50	50
Fiber (agar-agar)[b]	50	50	50	50	50	50
Mineral mix[e]	40	40	40	40	40	40
Vitamin mix[f]	20	20	20	20	20	20
SO[g]	50	50				
CO[h]			50	50		
SA[i]					50	50

[a]The diets were isocaloric (16.80 MJ/kg) and given in powdered form. Diet abbreviations: SOC, 20% casein + 5% SO; SOd, 2% casein + 5% SO; COC, 20% casein + 5% CO; COd, 2% casein + 5% CO; SAC, 20% casein + 5% SA; SAd, 2% casein + 5% SA.

[b]Prolabo, Paris, France.

[c]Merck, Darmstadt, Germany.

[d]Ets Louis François, Saint Maur les Fossés, France.

[e]UAR 205 B (Villemoisson, Epinay/Orge, France). Mineral mix provided the following (g/kg diet): calcium, 4; potassium, 2.4; sodium, 1.6; magnesium, 0.4; iron, 0.12; trace elements: manganese, 0.032; copper, 0.005; zinc, 0.018; cobalt, 0.00004; iodine, 0.00002.

[f]UAR 200 (Villemoisson). Vitamin mix provided the following (mg/kg diet): thiamin, 40; riboflavin, 30; nicotinic acid, 140; pyridoxine, 20; pyridoxal, 300; cyanocobalamin, 0.1; ascorbic acid, 1600; α-tocopherol, 340; menadione, 80; calcium panthotenate, 200; choline, 2720; folic acid, 10; *p*-aminobenzoic acid, 100; biotin, 0.6; retinol, 12; cholecalciferol, 0.125.

[g]Lesieur, Boulogne Billancourt, France.

[h]Astra Calvé, Asniere, France.

[i]Gattefossé, Saint Priest, France.

TABLE 2

Fatty Acid Composition (%) of Dietary Oils[a]

Fatty acid	SO	CO	SA
8:0	n.d.	4.0	n.d.
10:0	n.d.	6.0	n.d.
12:0	n.d.	39.0	n.d.
14:0	traces	19.0	2.0
16:0	10.3	16.0	16.0
16:1n-7	0.1	n.d.	6.0
18:0	3.9	4.6	4.0
18:1(n-7 + n-9)	22.1	8.9	18.0
18:2n-6	54.8	1.6	4.0
18:3n-3	7.5	n.d.	n.d.
20:0	0.4	n.d.	n.d.
20:1(n-9 + n-11)	0.2	n.d.	10.0
20:4n-6	n.d.	n.d.	1.1
20:5n-3	n.d.	n.d.	9.9
22:0	0.4	n.d.	n.d.
22:1(n-9 + n-11)	n.d.	n.d.	8.0
22:5n-3	traces	n.d.	3.9
22:6n-3	n.d.	n.d.	11.1
24:0	traces	n.d.	n.d.
24:1n-9	n.d.	n.d.	1.2
Saturated	15.0	88.6	22.0
PUFA (n-6 + n-3)	62.3	1.6	30.0
PUFA/saturated ratio	4.2	0.02	1.4

[a]Traces, <0.1%; n.d., "not detected." PUFA, polyunsaturated fatty acids. See Table 1 for other abbreviations.

Blood samples. On day 28 of the experiment, after overnight fasting, rats were bled from the abdominal aorta under anesthesia (sodium pentobarbital, 60 mg/kg body weight). Serum was obtained by low-speed centrifugation and preserved with 0.26 mmol disodium ethylenediaminetetraacetic (EDTA) and 3 mmol sodium azide.

Isolation of VLDL fraction from total lipoproteins. The VLDL fraction was isolated by a single spin discontinuous gradient following the method of Redgrave *et al.* (12) as modified by Meghelli-Bouchenak *et al.* (13). The purified VLDL fraction (d < 1.006) was dialyzed against 0.15 M NaCl, 1 mM EDTA, pH 7.4, at 4° for 24 h, using Spectra/Por 2 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA).

Apolipoprotein (apo) electrophoresis. After partial delipidation, VLDL-apos were estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Meghelli-Bouchenak *et al.* (13). Linear gradient acrylamide gels (2.5–20% acrylamide) were poured, using two chamber gravity flow gradient markers (14). Electrophoresis was performed in an LKB 2001-001 vertical electrophoresis unit (LKB, Bromma, Sweden) at 4°C for 18 h with 20 mA/slab gel. After staining with Coomassie brilliant blue G 250, destained gels were scanned at 600 nm with a densitometer (Model Profil 26; Sebia, Issy les Moulineaux, France). The proportions of the various apo were determined semiquantitatively

based on the densitometry tracings. The concentration of each apo was calculated based on the percentage of the area for each apo relative to the total area for each serum sample. Data are expressed as arbitrary units (AU)/L serum. The apo samples for the six groups were electrophoresed in parallel, but staining affinity of each peptide was not determined. However, when 50–200 μg of total protein were applied, the chromo-genicity of each major band was shown to change linearly relative to the amount of total protein applied to the gel. In order to affirm the validity of our calculations, immunoelectrophoresis according to Laurell (15) was also used for apo A-I, apo A-IV and total apo B quantitation.

Livers. The animals were killed, and the livers were removed, washed with cold saline, quickly blotted and weighed. About 1 g of the largest lobe was homogenized in an ultraturax homogenizer (Bioblock Scientific, Illkirch, France) for lipid extraction. About 100 mg of the same lobe was homogenized at 4°C in a Potter Elvejhem homogenizer and used for protein determination.

Chemical analyses. Total lipids of VLDL and liver were extracted according to the method of Folch *et al.* (16). Lipid classes were isolated by thin-layer chromatography (TLC) according to the method of Stahl *et al.* (17). The solvents used for lipid extraction and TLC contained 2,6-di-*tert*-butyl-*p*-cresol (50 mg/L) as antioxidant, and the lipid extracts were stored under nitrogen in the dark at -20°C to prevent oxidation.

Internal standards [17:0 fatty acid, di-17:0 phosphatidylcholine, tri-17:0 triacylglycerol (TG) and 17:0 cholesteryl ester] were added to the samples prior to extraction. Phospholipids (PL) were quantified by measuring their phosphorus contents by the Bartlett method (18). PL and TG fractions were transmethylated and fatty acids were analyzed by gas–liquid chromatography (19). The Becker Gas Chromatograph Packard 417 (Downers Grove, IL) used was equipped with a glass capillary column, 39 m × 0.3 mm i.d., coated with carbowax 20M as stationary phase. The flow rate (H_2) was 6 mL/min, the inlet temperature was 202°C, and the detector temperature was 240°C, using pentadecanoic acid methyl ester as internal standard. Identification of fatty acids was by comparison of relative retention times with those of commercial standards. Peak areas were calculated with an ENICA 21 integrator (Delsi Instrument, Suresnes, France). The average molecular weight of the fatty acids was determined (after correction for recovery relative to 17:0 internal standards) based on which the amounts of TG were calculated. Proteins were determined according to Lowry *et al.* (20) using bovine serum albumin as standard.

Statistical analysis. Results are reported as means for each group with standard errors of the means. In fatty acid analysis, when percentage data were not normally distributed, statistical analyses were carried out using the variance-equalizing transformation (arcsin transformation) according to Zar (21). Student's *t*-test was used to determine the significance of differences between pairs of particular means (2% vs. 20% casein). The effects of dietary fat, protein level and any interaction between the two (interaction "protein* oil"), when the CO and SA diets were compared to the SO diets (controls), were assessed by means of a two-way analysis of variance. The level of significance was 0.05.

RESULTS AND DISCUSSION

Animals. The daily average food intake per rat was 20.5 ± 4.8, 9.9 ± 1.5, 20.5 ± 4.8, 9.9 ± 1.2, 22.0 ± 4.4 and 10.1 ± 1.5 g/24 h for SOC, SOd, COC, COd, SAC and SAd, respectively. After 28 d, body weight was significantly affected by the level of protein in the diet. The protein-deficient groups, SOd, COd and SAd, weighed only 49, 43 and 44% ($P < 0.001$) of their respective controls. With the 20% casein diets, no difference was observed in body weights (293 ± 8, 286 ± 11 and 291 ± 46 g) for COC, SAC and SOC, respectively, independent of the oil in diet, which may have been due to the short experiment time.

Liver and VLDL TG and PL contents. The liver weights (Table 3) were lower in rats fed the low protein diets compared to their respective controls, i.e., SOd (–38%), COd (-40%) and SAd (–52%) (2% vs. 20% casein, $P < 0.001$), but there was no effect of the dietary fat source on liver weight. In protein-deficient rats, total hepatic lipids were markedly elevated as had been observed in previous experiments by Meghelli-Bouchenak *et al.* (10). A gain in liver total lipids, which was mostly due to TG increases, was observed in all protein-deficient groups ($P < 0.001$) (Table 3). This lipid accumulation in liver was attributed by Flores *et al.* (22) and Meghelli-Bouchenak *et al.* (13) to impaired hepatic TG secretion *via* VLDL. Yet, as liver TG levels (per gram of liver) were increased by protein malnutrition, PL levels in both liver and VLDL were decreased, especially in the CO-fed group, but not significantly for liver–PL in the SA-fed group. The highest liver–TG contents were observed with the CO diets ($P < 0.001$). As protein deficiency induced a reduction in VLDL-apo synthesis, TG levels carried by VLDL were decreased (Table 3).

Liver and VLDL fatty acid changes. PUFA occur in tissues as essential components of structural lipids of cellular and subcellular membranes. The properties of these membranes are affected by the PUFA composition of their constituent PL. Thus, PUFA status is best measured by the percentage of PUFA in the total fatty acids bound in PL, rather than by the overall PUFA concentration in tissues or fluids (23). In the present study, when comparing dietary variables, we have only considered the relative percentages, per weight, and not the actual amounts of fatty acids present. The fatty acid compositions of liver and VLDL lipids (expressed as percentage of total fatty acids) from rats on the different treatments are listed in Tables 4–7. In general, the fatty acid profiles of the lipid fractions studied reflected the fatty acid contents of the oils ingested by the various groups, particularly with 20% rather than with 2% casein in the diets. Thus, linoleic and arachidonic acid levels were highest in lipids from the SO-fed groups, and lowest in the SA- and CO-fed groups, reflecting the differences in the linoleic acid contents of the oils. Similarly, the long-chain n-3 PUFA were not detectable in

TABLE 3

Protein and Lipid Contents of Liver and VLDL[a]

	SO diets		CO diets		SA diets		Oil effect[b]		Protein effect[c]		Protein* oil[d]	
	SOC	SOd	COC	COd	SAC	SAd	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO
Liver												
Liver weight (g)	8.8 ± 0.4	5.5 ± 0.1	9.0 ± 0.2	5.4 ± 0.2	9.8 ± 0.4	4.7 ± 0.2	NS	NS	<0.001	<0.001	NS	0.007
Protein (mg/g liver)	181 ± 12	158 ± 8	202 ± 7	167 ± 13	238 ± 13	211 ± 8	NS	<0.001	0.010	0.027	NS	NS
Total lipid (mg/g liver)	46 ± 3	112 ± 12	68.3 ± 1.4	145 ± 13	58.7 ± 4.4	104.0 ± 11	0.006	NS	<0.001	<0.001	NS	NS
Phospholipid (mg/g liver)	37.0 ± 3.1	32.0 ± 1.1	41.8 ± 1.1	27.9 ± 1.8	37.6 ± 2.3	32.5 ± 3.1	NS	NS	<0.001	NS	0.033	NS
Triacylglycerol (mg/g liver)	4.6 ± 0.3	42.2 ± 2.7	18.3 ± 0.7	80.3 ± 2.7	14.6 ± 0.9	46.6 ± 1.4	<0.001	<0.001	<0.001	<0.001	<0.001	NS
VLDL												
Protein (g/L serum)	0.30 ± 0.08	0.10 ± 0.03	0.26 ± 0.02	0.17 ± 0.01	0.21 ± 0.02	0.14 ± 0.02	NS	NS	<0.001	0.003	NS	NS
Phospholipid (mmol/L serum)	8.3 ± 1.2	3.4 ± 0.5	5.4 ± 0.4	3.1 ± 0.3	3.8 ± 0.5	3.1 ± 0.3	0.031	0.002	<0.001	<0.001	NS	0.007
Triacylglycerol (mmol/L serum)	1.09 ± 0.10	0.45 ± 0.03	1.14 ± 0.38	0.60 ± 0.04	0.50 ± 0.03	0.24 ± 0.02	NS	<0.001	0.007	<0.001	NS	0.001

[a]Values are means for six rats ± SEM; abbreviations are as in Table 1 and VLDL, very low density lipoprotein. [b]*P*-values for hydrogenated coconut oil (CO) or salmon oil (SA) vs. soybean oil (SO). [c]*P*-values for 2% casein vs. 20% casein. [d]*P*-values for the interaction between oil type effect and protein levels; NS, nonsignificant.

these lipid fractions when rats were fed the CO diets and when the 22:5n-6 was replaced by DHA in rats fed the SA diets.

The amounts of total n-6 PUFA, i.e., linoleic and arachidonic acids, were lower with the CO and SA diets when compared to control values obtained with the SO diets ($P < 0.001$). The 20:3n-9/20:4n-6 ratio, a biochemical index of EFA deficiency (limit of normality is <0.2 in PL of serum) (23) was enhanced with the CO and SA diets (low in EFA), but more markedly with the coconut oil diet ($P < 0.001$). Yet, this ratio was not always increased in each of the lipid fractions by protein deficiency. Holman *et al.* (6) and Naismith (24) observed a significant increase in this ratio of plasma lipids of malnourished children, which was attributed to a moderate EFA deficiency. Koletzko *et al.* (3) found that the plasma lipids of malnourished children showed an EFA deficiency with only a slight rise in the triene/tetraene ratio in TG, but not in PL. These authors concluded that the ratio is not necessarily a reliable indicator of EFA status in protein malnutrition. Some studies on erythrocyte membrane lipids in malnourished children also showed lower proportions of eicosatrienoic acid (20:3n-9). Furthermore, one of these studies (7) showed low levels of linoleic and arachidonic acids in kwashiorkor and or arachidonic alone in marasmus; another study (25) reported lower proportions of arachidonic in the marasmic group than in the kwashiorkor group, and a higher unsaturated/saturated fatty acids (SFA) ratio in the kwashiorkor group than in the control group. These results are inconsistent, which may be due to the different degrees and duration of protein malnutrition and perhaps due to different dietary fat sources. The polyunsaturated/saturated (P/S) ratio was always depressed by protein deficiency in TG and PL of both liver and VLDL

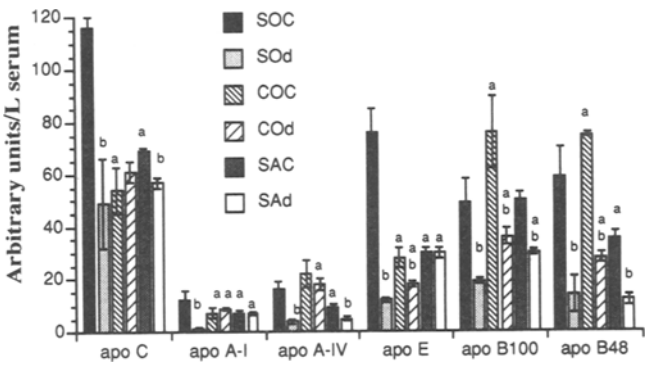

FIG. 1. Very low density lipoprotein-apolipoprotein (apo) distribution in young rats fed protein-deficient diets with coconut (CO) or salmon (SA) oils vs. soybean oil (SO), for 28 d. Values are expressed as arbitrary units (AU)/L. Serum values are means ± SEM for six rats per group. SOC, 20% casein + 5% SO; SOd, 2% casein + 5% SO; COC, 20% casein + 5% CO; COd, 2% casein + 5% CO; SAC, 20% casein + 5% SA; SAd, 2% casein + 5% SA. The level of significance, *P*, was determined by a *t*-test against the corresponding control; a or b indicates values with significant differences from controls with a *P*-value of at least $P < 0.05$; a, CO or SA vs. SO (COC or SAC vs. SOC and COd or SAd vs. SOd); b, 2% vs. 20% casein (COd vs. COC, or SAd vs. SAC, or SOd vs. SOC).

TABLE 4

Composition in Major Fatty Acids of Liver Triacylglycerols[a]

Major fatty acids	SOC	SOd	COC	COd	SAC	SAd	Oil effect[b]		Protein effect[c]		Protein* oil[d]	
							CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO
Total SFA	52.0 ± 1.7	51.5 ± 2.4	33.8 ± 3.8	37.7 ± 4.4	22.3 ± 2.3	25.2 ± 1.3	<0.001	<0.001	NS	NS	NS	NS
Total MUFA	28.2 ± 1.8	33.2 ± 1.4	52.8 ± 4.4	53.4 ± 5.0	31.3 ± 2.4	26.7 ± 2.0	<0.001	NS	NS	NS	NS	0.022
20:3n-9	0.3 ± 0.1	0.9 ± 0.2	2.8 ± 0.4	1.9 ± 0.7	2.8 ± 0.6	3.0 ± 0.5	<0.001	<0.001	NS	NS	NS	NS
18:2n-6	10.8 ± 1.7	6.2 ± 1.1	3.9 ± 0.3	2.7 ± 0.2	3.9 ± 0.3	4.5 ± 0.4	<0.001	<0.001	0.011	NS	NS	0.007
20:4n-6	2.4 ± 0.4	1.7 ± 0.4	1.0 ± 0.3	0.8 ± 0.2	2.1 ± 0.2	1.9 ± 0.1	0.002	NS	NS	NS	NS	NS
22:5n-6	0.7 ± 0.2	1.1 ± 0.3	4.2 ± 0.1	2.9 ± 0.2	traces	traces	<0.001	NS	0.044	NS	<0.001	NS
Total n-6 PUFA	13.9 ± 2.3	9.0 ± 1.8	9.1 ± 0.7	6.4 ± 0.6	6.0 ± 0.5	6.4 ± 0.5	0.025	0.002	0.022	NS	NS	NS
18:3n-3	n.d.	n.d.	n.d.	n.d.	2.7 ± 0.4	1.1 ± 0.3	—	—	—	—	—	—
20:5n-3	1.3 ± 0.1	0.3 ± 0.1	n.d.	n.d.	10.6 ± 0.9	3.9 ± 0.2	—	<0.001	—	<0.001	—	<0.001
22:5n-3	1.1 ± 0.2	2.7 ± 0.6	n.d.	n.d.	5.4 ± 0.3	6.8 ± 0.3	—	<0.001	—	<0.001	—	NS
22:6n-3	1.3 ± 0.1	0.3 ± 0.1	n.d.	n.d.	18.0 ± 2.1	26.5 ± 1.5	—	<0.001	—	0.008	—	0.001
Toal n-3 PUFA	3.7 ± 0.4	3.3 ± 0.8	n.d.	n.d.	36.7 ± 3.7	38.3 ± 3.7	—	<0.001	—	NS	—	NS
P/S[e]	0.34 ± 0.04	0.25 ± 0.02	0.35 ± 0.05	0.22 ± 0.01	2.04 ± 0.06	1.89 ± 0.07	NS	<0.001	0.001	0.026	NS	NS
20:3n-9/20:4n-6	0.12 ± 0.03	0.53 ± 0.07	2.80 ± 0.22	2.37 ± 0.19	1.33 ± 0.11	1.57 ± 0.09	<0.001	<0.001	NS	<0.001	NS	NS

[a]Values are means for six rats ± SEM; abbreviations are as in Table 1 and SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. [b]*P*-values for CO or SA vs. SO. [c]*P*-values for 2% casein vs. 20% casein. [d]*P*-values for the interaction between oil type effect and protein levels. [e]Polyunsaturated/saturated fatty acid ratio; n.d., not detected; traces, <0.1; NS, nonsignificant.

TABLE 5

Composition in Major Fatty Acids of Liver Phospholipids[a]

Major fatty acids	SOC	SOd	COC	COd	SAC	SAd	Oil effect[b]		Protein effect[c]		Protein* oil[d]	
							CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO
Total SFA	49.9 ± 4.4	52.2 ± 3.8	50.9 ± 3.6	64.4 ± 4.7	31.2 ± 2.4	32.6 ± 2.9	NS	<0.001	NS	NS	NS	NS
Total MUFA	14.8 ± 2.1	18.4 ± 1.4	20.9 ± 2.2	17.5 ± 1.2	20.5 ± 1.8	28.3 ± 1.9	NS	<0.001	NS	0.005	NS	NS
20:3n-9	n.d.	1.3 ± 0.5	16.9 ± 1.2	13.9 ± 0.8	9.2 ± 0.8	8.6 ± 0.7	<0.001	<0.001	NS	NS	0.010	NS
18:2n-6	9.1 ± 1.9	5.3 ± 1.1	3.3 ± 0.2	1.1 ± 0.2	4.5 ± 0.2	3.3 ± 0.2	0.002	0.031	0.013	0.034	NS	NS
20:4n-6	18.6 ± 1.4	14.2 ± 0.6	2.4 ± 0.4	1.1 ± 0.1	2.0 ± 0.2	2.1 ± 0.1	<0.001	<0.001	0.001	0.011	NS	0.008
22:5n-6	0.9 ± 0.2	1.7 ± 0.4	2.8 ± 0.2	0.7 ± 0.1	n.d.	n.d.	NS	NS	0.017	NS	<0.001	NS
Total n-6 PUFA	29.6 ± 3.6	21.3 ± 3.1	8.5 ± 0.8	2.8 ± 0.4	6.5 ± 0.4	5.4 ± 0.3	<0.001	<0.001	0.009	NS	NS	NS
18:3n-3	n.d.	n.d.	n.d.	n.d.	traces	traces	—	—	—	—	—	—
20:5n-3	0.2 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	traces	16.6 ± 0.7	2.7 ± 0.2	NS	<0.001	NS	<0.001	NS	<0.001
22:5n-3	3.2 ± 0.7	3.0 ± 1.0	n.d.	n.d.	2.5 ± 0.5	3.9 ± 0.1	—	NS	—	NS	—	NS
22:6n-3	2.3 ± 0.4	1.9 ± 0.3	1.7 ± 0.1	traces	12.0 ± 1.4	16.8 ± 0.8	<0.001	<0.001	<0.001	0.016	0.017	0.005
Total n-3 PUFA	5.7 ± 1.1	5.0 ± 1.3	2.6 ± 0.2	traces	31.1 ± 2.6	23.4 ± 1.1	<0.001	<0.001	0.023	0.019	NS	0.046
P/S[e]	0.71 ± 0.08	0.53 ± 0.07	0.55 ± 0.03	0.26 ± 0.01	1.50 ± 0.08	1.10 ± 0.06	<0.001	<0.001	<0.001	<0.001	NS	NS
20:3n-9/20:4n-6	—	0.09 ± 0.01	7.04 ± 1.12	13.9 ± 2.31	4.60 ± 0.26	4.09 ± 0.22	<0.001	<0.001	0.013	NS	0.015	NS

[a]Values are means for six rats ± SEM; abbreviations are as in Tables 1 and 4. [b]*P*-values for CO or SA vs. SO. [c]*P*-values for 2% casein vs. 20% casein. [d]*P*-values for the interaction between oil type effect and protein levels. [e]Polyunsaturated/saturated fatty acid ratio; n.d., not detected; traces, <0.1; NS, nonsignificant.

TABLE 6

Composition in Major Fatty Acids of VLDL Triacylglycerols[a]

Major fatty acids	SOC	SOd	COC	COd	SAC	SAd	Oil effect[b]		Protein effect[c]		Protein* oil[d]	
							CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO
Total SFA	32.2 ± 4.4	43.1 ± 5.2	44.6 ± 2.8	55.6 ± 2.8	34.0 ± 2.5	43.2 ± 2.7	0.004	NS	0.011	0.017	NS	NS
Total MUFA	43.4 ± 4.5	43.3 ± 3.8	43.6 ± 3.9	35.2 ± 2.9	23.3 ± 1.8	21.9 ± 2.2	NS	<0.001	NS	NS	NS	NS
20:3n-9	n.d.	n.d.	0.9 ± 0.2	1.2 ± 0.2	0.6 ± 0.2	0.9 ± 0.3	<0.001	<0.001	NS	NS	NS	NS
18:2n-6	14.0 ± 2.7	4.9 ± 1.3	5.2 ± 0.7	2.5 ± 0.2	2.4 ± 0.1	2.2 ± 0.2	<0.001	<0.001	0.001	0.005	NS	NS
20:4n-6	2.5 ± 0.3	1.8 ± 0.2	4.2 ± 0.4	2.6 ± 0.3	2.7 ± 0.3	2.6 ± 0.1	<0.001	0.049	0.001	NS	NS	NS
22:5n-6	2.1 ± 0.6	2.1 ± 0.6	0.9 ± 0.1	2.0 ± 0.4	n.d.	n.d.	NS	—	NS	—	NS	—
Total n-6 PUFA	19.5 ± 3.7	9.4 ± 2.5	10.3 ± 1.2	7.1 ± 0.7	5.1 ± 0.6	4.8 ± 0.6	0.023	<0.001	0.010	0.033	NS	0.043
18:3n-3	1.1 ± 0.3	1.3 ± 0.1	n.d.	n.d.	2.5 ± 0.3	1.0 ± 0.1	—	0.021	—	0.007	—	<0.001
20:5n-3	1.0 ± 0.4	0.8 ± 0.0	n.d.	n.d.	15.2 ± 1.2	10.4 ± 1.0	—	<0.001	—	0.005	—	0.010
22:5n-3	0.5 ± 0.1	traces	n.d.	n.d.	4.8 ± 0.4	2.7 ± 0.3	—	<0.001	—	<0.001	—	0.004
22:6n-3	2.1 ± 0.6	1.2 ± 0.2	n.d.	n.d.	12.9 ± 0.6	15.0 ± 0.9	—	<0.001	—	NS	—	0.027
Total n-3 PUFA	4.7 ± 1.4	3.3 ± 0.3	n.d.	n.d.	35.4 ± 2.5	29.1 ± 2.3	—	<0.001	—	0.049	—	NS
P/S[e]	0.82 ± 0.03	0.36 ± 0.06	0.25 ± 0.02	0.15 ± 0.01	1.27 ± 0.23	0.80 ± 0.07	<0.001	0.001	<0.001	<0.001	<0.001	NS
20:3n-9/20:4n-6	—	—	0.21 ± 0.03	0.46 ± 0.05	0.22 ± 0.04	0.34 ± 0.06	<0.001	<0.001	<0.001	<0.001	<0.001	0.005

[a]Values are means for six rats ± SEM; abbreviations are as in Tables 1 and 4. [b]*P*-values for CO or SA vs. SO. [c]*P*-values for 2% casein vs. 20% casein. [d]*P*-values for the interaction between oil type effect and protein levels. [e]Polyunsaturated/saturated fatty acid ratio; n.d., not detected; traces <0.1; NS, nonsignificant.

TABLE 7

Composition in Major Fatty Acids of VLDL Phospholipids[a]

Major fatty acids	SOC	SOd	COC	COd	SAC	SAd	Oil effect[b]		Protein effect[c]		Protein* oil[d]	
							CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO	CO vs. SO	SA vs. SO
Total SFA	42.5 ± 3.3	47.3 ± 6.3	47.6 ± 4.8	52.8 ± 4.3	34.2 ± 2.5	41.5 ± 3.5	NS	NS	NS	NS	NS	NS
Total MUFA	18.4 ± 3.5	22.5 ± 4.0	32.1 ± 1.4	24.3 ± 1.4	14.7 ± 0.9	18.3 ± 1.4	0.012	NS	NS	NS	0.048	NS
20:3n-9	n.d.	n.d.	5.8 ± 0.4	8.7 ± 0.7	n.d.	n.d.	—	—	—	—	—	—
18:2n-6	16.1 ± 1.9	11.7 ± 1.2	6.9 ± 0.4	4.3 ± 0.3	8.9 ± 1.2	5.9 ± 1.0	<0.001	<0.001	0.006	0.013	NS	NS
20:4n-6	15.1 ± 2.2	10.0 ± 2.2	4.9 ± 0.2	3.7 ± 0.1	12.7 ± 1.1	5.3 ± 0.7	<0.001	0.047	0.049	0.001	NS	NS
22:5n-6	2.1 ± 0.4	2.2 ± 0.4	2.2 ± 0.2	4.6 ± 0.6	n.d.	n.d.	0.007	0.008	0.012	NS	NS	NS
Total n-6 PUFA	33.3 ± 3.2	23.9 ± 1.7	14.0 ± 1.0	12.6 ± 1.2	21.6 ± 2.3	11.2 ± 1.7	<0.001	<0.001	0.015	<0.001	NS	NS
18:3n-3	n.d.	n.d.	n.d.	n.d.	1.0 ± 0.1	0.6 ± 0.1	—	—	—	—	—	—
20:5n-3	n.d.	n.d.	n.d.	n.d.	13.0 ± 1.1	9.1 ± 0.5	—	—	—	—	—	—
22:5n-3	2.6 ± 0.7	1.4 ± 0.7	n.d.	n.d.	3.7 ± 0.2	1.3 ± 0.6	—	NS	—	0.005	—	NS
22:6n-3	2.8 ± 1.1	2.2 ± 0.5	n.d.	n.d.	11.2 ± 1.0	15.8 ± 0.9	—	<0.001	—	0.002	—	0.009
Total n-3 PUFA	5.4 ± 0.4	3.6 ± 0.3	n.d.	n.d.	28.9 ± 2.4	26.8 ± 2.1	—	<0.001	—	NS	—	NS
P/S[e]	0.95 ± 0.15	0.62 ± 0.02	0.42 ± 0.04	0.40 ± 0.02	1.47 ± 0.16	0.93 ± 0.09	<0.001	0.002	0.046	0.001	NS	NS
20:3n-9/20:4n-6	—	—	1.18 ± 0.21	2.35 ± 0.23	—	—	—	—	—	—	—	—

[a]Values are means for six rats ± SEM; abbreviations are as in Tables 1 and 4. [b]*P*-values for CO or SA vs. SO. [c]*P*-values for 2% casein vs. 20% casein. [d]*P*-values for the interaction between oil type effect and protein levels. [e]Polyunsaturated/saturated fatty acid ratio; n.d., not detected; traces, <0.1; NS, nonsignificant.

($P < 0.001$), and the highest ratio was always obtained with the SA diets ($P < 0.001$).

Thus, protein malnutrition does not have the same effects on the fatty acid composition with using the SA diets or the CO diets. In VLDL–PL (Table 7), protein deficiency diminished total monounsaturated fatty acids (MUFA) with the CO diets but raised them with the SA diets. The total amounts of n-6 fatty acids, i.e., 18:2n-6 (not significantly for SAd) and 20:4n-6, were diminished in both liver- and VLDL–PL of rats fed the protein-deficient diets independent of the oil (Tables 5 and 7).

Hill and Holman (4) reported that when CO is fed at various protein levels in the diet, PUFA profiles of liver–PL showed significantly lower amounts of n-6 fatty acids at low levels of protein, except for the amount of 22:4n-6, which was significantly higher. In our study, "protein* oil" interaction was seen in regard to the elongation products of the n-6 (with the CO diets) and n-3 (with the SA diets) series. Low protein levels together with coconut oil lowered the amounts of 22:5n-6 in liver lipids (TG + PL) ($P < 0.001$), but the amounts of 22:5n-6 were elevated in VLDL lipids. In previous studies using young rats, we have shown that in α-linolenic acid deficiency and with adequate linoleate intake, protein deficiency increased the amount of 22:5n-6 in VLDL–PL (26, 27). This fatty acid seemed to be secreted to a greater extent when both protein and n-3 fatty acid deficiencies were present. The increased amount of 22:5n-6 in VLDL could also be attributable to a lower utilization of this acid by tissues other than liver. Similarly, the SAd group showed reduced amounts of total n-3 fatty acids in liver–PL and VLDL–TG, but despite the lower amounts of 20:5n-3, 22:6n-3 was increased in all lipid fractions (interaction protein* salmon oil). We have previously observed the same phenomenon in the PL of liver microsomes from rats fed similar diets (28). The data may indicate that the conversion of EPA to DHA is accelerated by protein malnutrition with the SA diets.

VLDL apo. The CO diets enhanced apo B100, apo B48 and apo A-IV more effectively than either the SO or the SA diets (Fig. 1). Total VLDL-apo were reduced by protein deficiency (Table 3), probably because of reduced synthesis as was also observed by Yagasaki and Kametaka (29) and Meghelli-Bouchenak *et al.* (10). Previous data indicate that the reduced synthesis by liver of VLDL-apo is a cause of impaired hepatic TG export, and thus fatty liver. Apo B and apo C, which are the main apo synthesized by the liver of rats, are particularly depressed by protein deficiency and sunflower oil diets (13–30). In the present work, total VLDL-apo were decreased in rats fed low protein diets independent of the dietary oil. The decline was mainly due to apo B100 and apo B48. Apo A-I was not decreased when feeding CO or SA oils, while all apo were decreased with SO and in protein deficiency. We have shown previously that protein deficiency reduces the half-lives of VLDL-apo and increases their clearance, probably because of increased peripheral uptake by different organs, but not due to a higher uptake by liver (31).

In conclusion, in spite of the short feeding period (28 d) used in the present study, protein malnutrition was shown to accentuate the impairment in PUFA supply, i.e., the P/S ratio was lowered in rats fed the low-protein diets. Yet, dependent on the type of oil used, the magnitude of the observed changes differed. In rats fed 20% casein, the fatty acid profiles of the various lipid fractions better reflected the fatty acid composition of the dietary oils, than in rats fed 2% casein. Protein deficiency severely diminished the amounts of linoleic, arachidonic and EPA acids, but did so to a lesser extent with SA than with CO. Conversely, despite decreased proportions of EPA at low protein levels, DHA remained higher in rats fed the SA diets, whereas in the CO-fed rats, the amount of 22:5n-6 was lower in liver but higher in VLDL lipids at low protein levels. Arachidonic acid and EPA are precursors of prostaglandins, thromboxanes and leukotrienes, and the possible consequences of their diminution due to protein malnutrition may be an underlying cause of some of the common clinical symptoms in kwashiorkor, such as hair loss, dry scaly dermatitis and increased water permeability.

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Blood Polyunsaturated Fatty Acids in Patients with Peroxisomal Disorders. A Multicenter Study

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The purpose of the study was to compare the polyunsaturated fatty acid (PUFA) status in patients with X-linked adrenoleukodystrophy or adrenomyeloneuropathy (X-ALD/AMN) with that in disorders of peroxisome biogenesis (PB). Total fatty acids and plasmalogens were quantified in plasma and red cells from 28 patients with X-ALD/AMN, 26 patients with generalized peroxisomal disorders, and 37 controls. Total fatty acid methyl esters and plasmalogen dimethyl acetals were obtained by direct transmethylation and separated by capillary column gas chromatography. The results confirm previous findings in that docosahexaenoic acid (DHA, 22:6n-3) was greatly decreased in both plasma and erythrocytes from patients with PB disorders. When nutritional conditions were adequate, patients with X-ALD/AMN had normal levels of DHA. A highly significant positive correlation was found between the levels of DHA and those of plasmalogens in peroxisomal patients. As in other tissues, the parent n-6 fatty acid, linoleic acid (LA, 18:2n-6) was significantly increased in red cells from PB patients, whereas arachidonic acid (20:4n-6) was virtually within normal limits. In clear contrast to red cells and other tissues, arachidonate was significantly lower in plasma from PB patients. The decrease in plasma arachidonate and the high tissue levels of LA suggest a defect of Δ6 desaturase and/or Δ5 desaturase in PB patients. The n-6 fatty acids were normal in X-ALD/AMN patients. The present data show that X-ALD/AMN patients do not have the profound PUFA alterations that PB patients have, at least in blood.

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Generalized peroxisomal disorders are a group of genetic diseases that are characterized by the absence or by a significant decrease in the number of peroxisomes, particularly in liver and kidney (1). The biogenesis of peroxisomes seems to be defective in these diseases, so that the name "disorders of peroxisome biogenesis" has recently been proposed (2). Various peroxisomal enzyme functions are impaired in this group of disorders. One of the impairments more consistently found is the defective β-oxidation of very long chain fatty acids (VLCFA), i.e., fatty acids with more than 22 carbon atoms, which leads to accumulation in tissues of 24:0, 26:0 and 26:1n-9 (3). Detection of VLCFA in plasma and skin fibroblasts in culture is widely used to diagnose peroxisomal disorders (4,5).

There are also various other functions associated with peroxisomes that are affected in peroxisomal disorders. Plasmalogen synthesis is altered (6) by a defect in dihydroxyacetonephosphate acyltransferase (7). Also, very often accumulation of phytanic acid (8) and bile acid intermediates (9) occurs, and abnormal excretion of dicarboxylic acids is observed (10). Classification of the disease is based on clinical symptoms and on the different enzyme functions that are affected, as well as on complementation analysis (11). The disease prototype, Zellweger's syndrome (ZS), is the most severe form of these disorders (12). In ZS, all peroxisomal functions are defective, and patients are profoundly affected from birth by characteristic craniofacial dysmorphias, hypotony, convulsions, hepatomegaly, mental retardation, and visual and auditory defects. Deterioration is very rapid, and the patients usually die totally demented during their first months of life. Microscopically, liver biopsies show the absence of peroxisomes. Post-mortem examination reveals dysmyelination and neuronal heterotopias.

Milder variants of ZS are neonatal adrenoleukodystrophy (NALD; 13) and infantile Refsum's disease (IRD; 14). However, classification is often arbitrary, and overlap occurs between the different clinical forms. Children with IRD and NALD are less severely affected than those with ZS, and they often live until their second decade. A related disease is X-linked adrenoleukodystrophy (X-ALD; 15). In this disorder, peroxisomes are present and microscopically normal, but β-oxidation of VLCFA is defective (16). The affected reaction seems to be the conversion of VLCFA to the corresponding acyl-CoA esters (17). The onset of the disease is at 5–8 years of age, followed by progressive deterioration of cerebral and motor functions leading to death within a few years. In a variant of X-ALD, adrenomyeloneuropathy (AMN), the spinal cord and the peripheral nervous system are affected (18). The disease does not manifest itself until adulthood, and the clinical symptoms are milder than in X-ALD. AMN patients live for several decades.

Although many studies have been undertaken, and a genetic defect in some X-ALD patients has now been pinpointed (19), the immediate cause of the neurological deterioration in these patients remains unknown. Phytanic acid toxicity has been associated with the adult form of Refsum's disease, but the levels of phytanate in IRD are usually much lower. Thus, much of the damage has been attributed to VLCFA, although these fatty acids are normal components of myelin at lower levels. Also, in patients with another peroxisomal disorder, rhizomelic chondrodysplasia punctata (20), β-oxidation of VLCFA is normal. The lower levels of plasmalogens could be an important factor, since plasmalogens are

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Abbreviations: AA, arachidonic acid; AMN, adrenomyeloneuropathy; DHA, docosahexaenoic acid; DMA, dimethyl acetals; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; IRD, infantile Refsum's disease; LA, linoleic acid; NALD, neonatal adrenoleukodystrophy; PB, peroxisome biogenesis; PUFA, polyunsaturated fatty acids; RCDP, rhizomelic chondrodysplasia punctata; VLCFA, very long chain fatty acids; X-ALD, X-linked adrenoleukodystrophy; ZS, Zellweger's syndrome.

abundant in the brain, especially in myelin. However, in pseudo-Zellweger's syndrome (21), a variant of ZS due to defective 3-oxoacyl-CoA thiolase (22), plasmalogen levels are normal, although the disease is very similar, and almost as severe as classic ZS.

Recently, a marked deficiency of docosahexaenoic acid (DHA, 22:6n-3) has been observed in brain, retina, liver and kidney from patients with defective peroxisome biogenesis (PB) (23–25). This DHA decrease can also be seen in plasma and erythrocytes (26). DHA is an important component of brain and retinal membrane phospholipids, and its deficiency has been associated with neurological and visual impairment in the rhesus monkey (27), as well as in the human infant (28). A decrease in brain DHA of the magnitude found in patients with defective peroxisomal biogenesis could be responsible for many of the symptoms in these patients. DHA synthesis in rat liver has been shown to occur by β-oxidation of the VLCFA 24:6n-3 (29). If this metabolic route is also prevalent in humans, patients with other β-oxidation defects, such as X-ALD/AMN patients, might also suffer from DHA deficiency (30). It was, therefore, of interest to assess whether DHA deficiency in patients with late onset peroxisomal disorders, such as X-ALD and AMN, was the same as that in patients with early onset disorders of PB. Our study shows that differences exist in polyunsaturated fatty acid (PUFA) composition in red cells and in the plasma of patients with disorders of PB and with X-ALD/AMN. For comparison, the PUFA compositions of omnivore and vegetarian controls are also presented.

MATERIALS AND METHODS

The study included 53 patients of different ages with different forms of peroxisomal disorders. The patients could be subdivided into two major groups. One group consisted of 28 patients with ages ranging from 5 to 44 yr, with X-ALD or AMN. The other group consisted of 25 patients with disorders of PB that ranged from extremely severe to relatively mild; these patients' ages were between 2 mon and 19 yr. All patients in this group had been diagnosed as having ZS, NALD or IRD. About 5 mL of blood was collected under fasting conditions, using ethylenediaminetetraacetic acid as anticoagulant. The sample was shipped on ice by express courier and usually arrived in the laboratory within 48 h of collection. Erythrocytes were separated from plasma and leukocytes on the day of arrival and washed twice with isotonic saline solution. When possible, erythrocytes and plasma were analyzed immediately. Otherwise, the two fractions were stored at 4°C for as short a period of time as possible. Freezing of erythrocytes was avoided since it damages the cell membranes and causes PUFA losses (31), as was confirmed in our laboratory.

Total acyl groups were quantified as methyl esters using 13:0 as internal standard. Plasmalogens were quantified, after conversion of the alkenyl group to the DMA (32) of the corresponding long chain aldehydes (mostly 16:0, 18:0 and 18:1 DMA). Total fatty acid methyl esters (FAME) and long-chain DMA were obtained by direct transmethylation (33) of red cell and plasma lipids with HCl/methanol as described (24). Briefly, 2 mL of methanol/benzene (4:1, vol/vol) containing 100 μg of tridecanoic acid (13:0) was added to 100 μL of plasma or washed red cells in a methanolysis tube (Corning, NY). The tube was vigorously vortexed, while 0.2 mL of acetyl chloride was slowly added. The tube was tightly stoppered and then placed into a heating block (Thermolyne 7600 Dri-Bath, Dubuque, IA) at 100°C for 1 h. After centrifugation at 4,000 rpm for 5 min, an aliquot of the upper phase (2–7 μL) containing FAME and DMA was analyzed by capillary column gas chromatography on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) with a 30 m × 0.25 mm i.d. SP-2330 column (Supelco, Bellefonte, PA). The carrier gas was helium at a column head pressure of 15 psi. Samples were injected at a split ratio of about 50:1, and the column was programmed from 140 to 200°C at 3°C/min. Using this program and separation conditions, all components including VLCFA and PUFA methyl esters as well as plasmalogen DMA were well separated. Injector and detector temperatures were 250°C. Detector response linearity was periodically checked with quantitative standard mixtures; recovery was found to be very good within the whole range of compounds studied.

Peak areas were measured with a Hitachi D-2000 computer-integrator (Hitachi Ltd., Tokyo, Japan), and components were identified by comparison of their retention times with those of pure standards and of mixtures with know composition. Peak assignments were confirmed by mass spectrometry of the DMA, methyl esters and/or picolinyl esters (34) using a Hewlett-Packard 5970B mass selective detector. Spectra were obtained at an ionization potential of 70 eV.

RESULTS

In confirmation of previous studies (23–26), patients with disorders of PB showed a marked decrease in the total concentration of DHA, 22:6n-3 both in plasma (Table 1 and Fig. 1) and in erythrocytes (Table 2 and Fig. 2). An exception was a child, who was only mildly affected neurologically and had virtually normal red cell DHA levels (MR in Fig. 1 and 2). Another child (NE in both figures) had an atypical variant of the disease, without dicarboxylic aciduria and had DHA levels in erythrocytes within normal range. In contrast to patients with PB disorders, all but two X-ALD/AMN patients had normal values of DHA in plasma as well as in red cells. The exceptions were two X-ALD patients (MSG and MC), who were fed by intubation on "elemental diets" (special, easily digestible mixtures used in patients that cannot be nourished normally, and composed of known quantities of purified defined elements, providing the basic nutritional requirements of carbohydrate, protein, fat vitamins and minerals), low in n-3 fatty acids.

The 22:6n-3/22:5n-3 ratio was typically low in PB patients, and normal in X-ALD/AMN patients, in plasma as well as in erythrocytes (Tables 1 and 2). Interestingly, normal and strictly vegetarian Indian children had

TABLE 1

Fatty Acid Composition of Plasma in Peroxisomal Patients and Controls

Fatty acid	Omnivore controls 1–58 yr (n = 27)	X-ALD/AMN 5–54 yr (n = 26)	X-ALD[a] 6 yr	X-ALD[a] 12 yr	PB disorders 2–19 yr (n = 25)	Vegetarian controls 7–10 yr (n = 4)
12:0	13.6 ± 9.1	14.8 ± 6.4	9.0	12.8	22.7 ± 22.2	26.3 ± 11.5
14:0	88.2 ± 45.6	141.8 ± 76.0	140.5	153.5	97.4 ± 53.2	181.7 ± 97.4
16:0	2603.8 ± 503.5	3191.8 ± 1093.3	3903.3	2627.2	2394.1 ± 676.2	2433.4 ± 924.0
16:1n-7	182.1 ± 82.1	256.4 ± 184.3	882.5	288.7	328.1 ± 449.0	162.1 ± 92.4
18:0	937.7 ± 201.5	1077.0 ± 236.4	1438.1	1339.7	728.2 ± 206.5	878.5 ± 186.1
18:1n-9	2704.9 ± 786.1	1341.9–9057.8	5921.7	3162.3	2214.4 ± 1046.9	2185.2 ± 1029.4
18:1n-7	201.7 ± 55.2	242.4 ± 102.6	232.2	174.8	117.3 ± 42.7	141.4 ± 48.7
18:2n-6	3389.8 ± 661.5	3704.2 ± 880.4	570.6	2687.0	2286.8 ± 816.0	3516.1 ± 824.4
18:3n-6	54.9 ± 20.1	75.3 ± 44.6	108.1	170.8	25.9 ± 14.1	47.8 ± 28.0
18:3n-3	34.2 ± 11.9	72.4 ± 69.2	5.6	48.1	37.3 ± 21.2	27.2 ± 11.2
20:0	37.0 ± 8.8	33.4 ± 11.3	35.6	29.8	22.9 ± 9.7	34.0 ± 7.2
20:1n-9	28.8 ± 28.3	52.7 ± 31.2	26.8	26.1	31.2 ± 11.7	31.3 ± 8.7
20:2n-6	37.9 ± 13.4	36.2 ± 11.1	38.5	52.6	32.2 ± 12.9	33.1 ± 8.0
20:3n-9	18.4 ± 9.8	35.4 ± 39.7	377.4	71.8	24.6 ± 24.9	18.5 ± 7.0
20:3n-6	217.1 ± 66.3	238.1 ± 89.9	441.6	703.9	108.6 ± 43.0	199.3 ± 73.3
20:4n-6	978.1 ± 206.6	951.2 ± 243.5	972.4	940.3	367.7 ± 144.1	892.8 ± 237.9
20:5n-3	66.4 ± 46.7	116.6 ± 82.0	8.6	87.6	18.7 ± 10.7	15.7 ± 7.1
22:0	92.5 ± 22.5	73.2 ± 22.0	79.5	75.4	46.8 ± 24.9	108.9 ± 12.1
22:1n-9	5.5 ± 3.9	1.5–1464.2	10.2	3.4	8.6 ± 5.7	2.3 ± 0.5
22:4n-6	30.0 ± 7.3	36.0 ± 14.3	108.2	77.1	35.0 ± 17.5	77.3 ± 13.1
22:5n-6	24.0 ± 9.2	20.8 ± 12.7	70.4	46.6	23.6 ± 12.0	55.6 ± 21.0
22:5n-3	47.0 ± 11.9	66.4 ± 25.6	17.8	76.7	24.4 ± 18.9	40.5 ± 9.2
22:6n-3	276.8 ± 104.9	308.8 ± 123.8	22.2	89.2	43.0 ± 36.7	74.1 ± 30.4
24:0	75.3 ± 16.1	111.0 ± 44.9	127.9	136.1	63.2 ± 28.8	117.0 ± 7.3
24:1n-9	142.7 ± 40.5	51.5–669.4	109.0	106.2	83.9 ± 39.7	92.0 ± 7.9
26:0	1.3 ± 0.4	4.9 ± 2.4	12.9	4.2	7.5 ± 3.5	2.2 ± 0.9
26:1n-9	1.0 ± 0.5	0.9–8.1	7.8	3.5	7.6 ± 6.1	0.4 ± 0.2
Phytanic	<5.0	<10.0	1.1	ND	0.2–218.4	<5.0
Total	12431 ± 2225	15630 ± 4525	15903	13462	9362 ± 2257	11533 ± 3523
24:0/22:0	0.822 ± 0.084	1.478 ± 0.313	1.609	1.804	1.354 ± 0.509	1.084 ± 0.114
24:1n-9/22:0	1.560 ± 0.346	0.945–9.349	1.371	1.408	2.210 ± 1.746	0.850 ± 0.074
26:0/22:0	0.015 ± 0.006	0.067 ± 0.025	0.162	0.055	0.224 ± 0.167	0.020 ± 0.008
26:1n-9/22:0	0.006 ± 0.007	0.013–0.196	0.098	0.046	0.429 ± 0.867	0.004 ± 0.002
22:6n-3/22:5n-3	5.97 ± 1.77	5.05 ± 2.07	1.244	1.16	1.85 ± 1.19	1.819 ± 0.535
22:5n-6/22:4n-6	0.80 ± 0.21	0.58 ± 0.23	0.650	0.60	0.71 ± 0.26	0.703 ± 0.131
20:4n-6/18:2n-6	0.30 ± 0.08	0.27 ± 0.08	1.704	0.35	0.17 ± 0.06	0.253 ± 0.012

[a]These two patients with X-linked adrenoleukodystrophy (X-ALD) were fed on elemental diets by intubation. The others had a more complete diet. The most characteristic fatty acid ratios that are usually altered in peroxisomal patients are displayed in the table. Fatty acids are given in nanomoles per mL of plasma. Grouped data are means ± 1 SD. The range, rather than the mean, is given for oleic and erucic acids, and for the longer derivatives 24:1 and 26:1 and their corresponding ratios to 22:0, because these values are very variable in X-ALD patients treated or not with monounsaturated oil mixtures (glyceryl trioleate/glyceryl trierucate). The same applies to phytanic acid that is very variable with age and diet in peroxisome biogenesis (PB) patients. AMN, adrenomyeloneuropathy.

levels of DHA almost as low as those in PB patients, and the 22:6n-3/22:5n-3 ratio was also reduced, as it was in X-ALD patients fed low PUFA diets. Like DHA, eicosapentaenoic acid (20:5n-3) was low in normal vegetarians, PB and malnourished X-ALD patients, and normal in well-nourished X-ALD/AMN patients.

As for the n-6 series in plasma from PB patients, linoleic acid (LA, 18:2n-6) was just below the normal limit, and arachidonic acid (AA, 20:4n-6) was markedly decreased (Table 1). In contrast to plasma, however, the LA levels in the red cells were significantly increased (Table 2), and AA levels were within normal limits. As found in other tissues (23–25), some n-6 intermediates, such as 20:3n-6 (dihomo-γ-linolenic acid), were slightly increased in red cells of PB patients and were normal in X-ALD/AMN patients, with the exception of the two malnourished children. In plasma from PB patients, AA and 20:3n-6 were both decreased, so that the ratio between the two was not much affected. Like in red cells, the 20:4n-6/18:2n-6 ratio was slightly decreased in the plasma from these patients.

In general, X-ALD/AMN patients had normal levels of both LA and AA in plasma as well as in red cells. The two X-ALD patients kept on elemental diets had low levels of LA, but AA was normal and the ratio between the two remained within normal limits. This was in contrast to PB patients, who had high LA and normal-to-low AA levels, with a 20:4n-6/18:2n-6 ratio close to 1.00 (Table 2). Vegetarian controls also had a high erythrocyte LA concentration, but, unlike PB patients, AA was also high so that a normal 20:4n-6/18:2n-6 ratio was maintained.

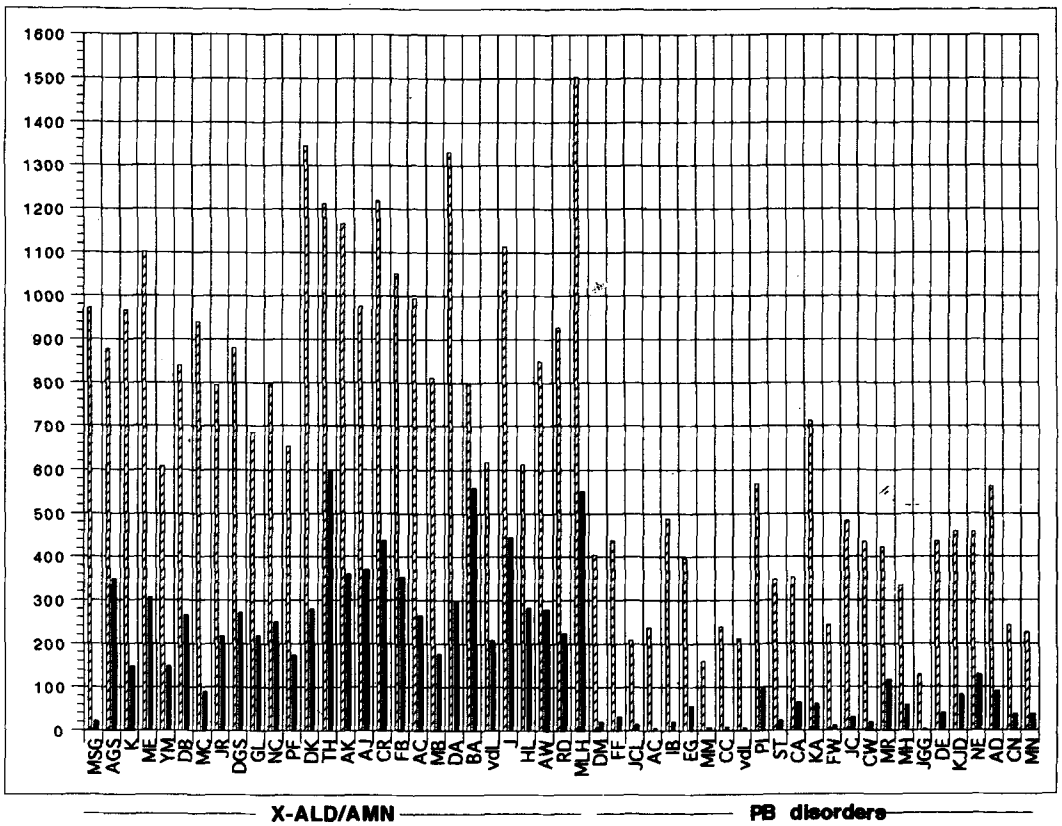

FIG. 1. Bars represent nmol/mL of docosahexaenoic (black bars) (DHA) and arachidonic (striped bars) (AA) acids in plasma of peroxisomal patients. Even with the wide individual variation found in plasma, a clear difference in DHA levels between X-linked adrenoleukodystrophy/ adrenomyeloneuropathy (X-ALD/AMN) patients and generalized peroxisomal patients is seen. The latter have very low DHA values, some are almost undetectable; the X-ALD/AMN patients have in general normal values, although their diets are very low in fat. The exceptions were two patients (first and seventh bar groups, MSG and MC) who were fed very low PUFA diets (see text). Plasma AA levels were also within normal limits in X-ALD/AMN patients and were low in peroxisome biogenesis (PB) patients.

Plasmalogen levels were normal in X-ALD/AMN patients on complete diets but decreased in the two patients on low PUFA diets (Tables 3 and 4). As found in peroxisomal patients under DHA therapy (26), a clear positive correlation was seen between the levels of DHA and those of total plasmalogens (Fig. 3), irrespective of the diagnostic group. Such a clear correlation was not found within the normal population (data not shown). In contrast to malnourished X-ALD/AMN and PB patients, vegetarian controls had normal red cell plasmalogen levels, despite their low blood DHA values (Tables 1 to 4).

DISCUSSION

Our data suggest that important differences exist in PUFA metabolism between patients with so-called defects of PB and those with late onset peroxisomal disorders, such as X-ALD and AMN. The major difference is in the DHA content, which is consistently low in red cells and plasma from patients with generalized peroxisomal disorders, and normal in well-nourished X-ALD/AMN patients. Interestingly, the only two X-ALD/AMN patients who showed DHA deficiency in red cells were the ones who received the "elemental diets" low in PUFA. The importance of nutrition and its effect on red cells PUFA patterns was reemphasized by the fact that normal children nourished on strictly vegetarian diets had levels of DHA almost as low as PB patients. In contrast to normal controls, however, the DHA deficiency was present in patients with defective PB, even when the diet was complete, and it was very pronounced in tissues, including brain and retina (25).

An interesting finding was the significant positive correlation between red cell DHA levels and plasmalogen levels, irrespective of disorders. This is consistent with observations made on monkeys fed fish oil (35) and on peroxisomal patients treated with DHA ethyl ester (26). The basis for such a relationship between DHA and plasmalogens is presently not clear. It has been known for some time, however, that ethanolamine plasmalogens are rich in PUFA (36), and selective reacylation of alkenyl lysophospholipids by DHA has been demonstrated in some cells (37). Therefore, it might be speculated that DHA is preferentially used in plasmalogen synthesis. This could also explain why plasmalogen levels in red cells were lower in some peroxisomal patients

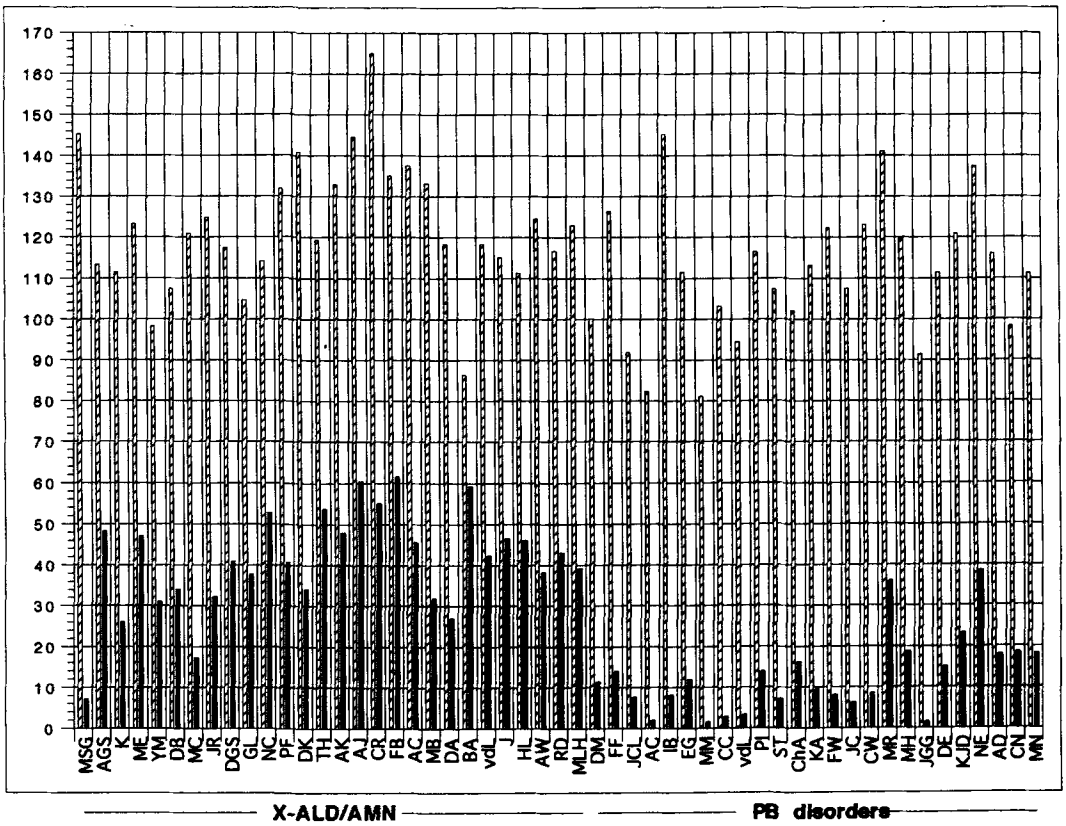

FIG. 2. Bars represent pmol/10^6 cells of docosahexaenoic (black bar) (DHA) and arachidonic (striped bar) (AA) acids in erythrocytes of peroxisomal patients. The red cell levels of DHA are notably low in PB patients, except for two cases (MR and NE) with atypical clinical pictures. In all but two X-ALD/AMN patients, DHA was normal. The two exceptions (MSG and MC) were patients with very low polyunsaturated fatty acid intakes. AA levels are within normal limits in all cases, in X-ALD/AMN as well as in PB patients. See Figure 1 for abbreviations.

while the activity of dihydroxyacetonephosphate acyltransferase in cultured skin fibroblasts was normal (26). The *in vitro* bovine medium, which is relatively rich in n-3 fatty acids, might provide enough substrate for plasmalogen synthesis in the fibroblasts *in vitro*, whereas *in vivo* the low DHA plasma levels seem insufficient to synthesize plasmalogens at a normal rate.

As for n-6 fatty acids, the differences were more subtle. In red cells from PB patients, as in other tissues (23–25), LA was slightly increased and AA was within normal limits; in well-nourished X-ALD/AMN patients, both LA and AA had normal values. An unexpected finding was a significant decrease in AA in plasma of PB patients, which was not detected in any other tissue. This is intriguing, and, together with the increase in LA present in most tissues (23–25), could indicate the possible coexistence of some other enzyme defects, such as low Δ6 or Δ5 desaturase activities. However, since plasma LA was decreased rather than increased, the low levels of AA could be simply due to nutritional deficiency. Peroxisomal patients are usually subjected to very strict diets, virtually devoid of fat and very low in long PUFA. Under these conditions, it does not seem surprising that all AA generated would be utilized for membrane phospholipid synthesis, especially in the brain and retina, with low AA levels remaining in plasma. In any case, it is clear that the AA deficiency, if at all present in tissues, is much less severe than the DHA deficiency, as indicated by the very low DHA/AA ratio in all tissues, including the retina (25). In the brain, in particular, AA was not decreased, and AA even was somewhat increased in ZS, quite in contrast to DHA.

It can be thus concluded that under adequate nutritional conditions X-ALD/AMN does not seem to significantly affect PUFA metabolism, whereas in disorders of PB DHA is consistently decreased; this decrease is clearly seen in erythrocytes. Our present results on red cells from X-ALD/AMN patients do not support the hypothesis that the cause of DHA deficiency in peroxisomal patients is defective β-oxidation of VLCFA (29,30). However, the study of the PUFA patterns in one X-ALD patient showed low brain DHA levels (25). The possibility that there is some defect in brain DHA metabolism that is not reflected in blood cannot be ruled out at the present time, and more X-ALD brains should be studied before drawing any definitive conclusion.

In PB patients, LA is generally high in red cells and in other tissues, but usually low in plasma. AA is within

TABLE 2

Total Fatty Acid Composition of Erythrocytes in Peroxisomal Patients and Controls

	Omnivore controls 4–58 yr (n = 33)	X-ALD/AMN 5–54 yr (n = 26)	X-ALD[a] 6 yr	X-ALD[a] 12 yr	PB disorders 2–19 yr (n = 25)	Vegetarian controls 5–8 yr (n = 4)
14:0	2.61 ± 0.68	3.17 ± 1.01	3.54	3.52	3.46 ± 1.56	4.83 ± 1.59
16:0	178.70 ± 21.04	176.17 ± 20.14	215.29	171.09	196.36 ± 24.42	180.71 ± 9.15
16:1n-7	1.96 ± 2.69	1.70 ± 0.88	9.55	1.83	3.79 ± 3.07	2.13 ± 1.05
18:0	140.30 ± 17.13	136.38 ± 14.65	156.08	152.55	130.45 ± 19.40	143.18 ± 7.46
18:1n-9	109.31 ± 16.30	116.27 ± 27.97	147.40	100.66	102.56 ± 27.13	97.46 ± 8.56
18:1n-7	8.59 ± 2.06	8.59 ± 2.08	10.68	6.94	7.34 ± 1.49	6.79 ± 0.99
18:2n-6	76.36 ± 14.64	74.09 ± 17.12	28.37	56.80	106.31 ± 27.06	105.84 ± 7.01
18:3n-6	0.41 ± 0.23	0.54 ± 0.24	1.19	0.81	0.61 ± 0.25	0.59 ± 0.44
18:3n-3	0.76 ± 0.52	0.89 ± 0.44	0.33	0.42	1.14 ± 0.55	0.54 ± 0.15
20:0	3.70 ± 0.78	2.82 ± 0.87	3.20	2.50	2.60 ± 0.77	3.98 ± 0.19
20:1n-9	2.44 ± 0.43	3.46 ± 1.50	1.95	2.85	3.23 ± 1.62	2.04 ± 0.28
20:2n-6	3.29 ± 1.19	2.46 ± 0.90	3.33	3.97	3.44 ± 1.26	2.82 ± 0.65
20:3n-9	0.87 ± 0.34	1.87 ± 1.40	10.24	1.64	2.70 ± 2.73	2.93 ± 0.54
20:3n-6	14.41 ± 2.99	14.41 ± 3.26	37.77	37.75	17.31 ± 6.84	14.81 ± 1.37
20:4n-6	125.97 ± 19.80	121.88 ± 15.91	145.15	120.94	111.04 ± 16.71	149.58 ± 8.41
20:5n-3	4.62 ± 2.52	5.93 ± 2.77	1.33	2.62	2.82 ± 1.40	1.69 ± 0.23
22:0	15.17 ± 3.03	12.52 ± 6.16	11.10	8.84	12.61 ± 4.73	20.32 ± 1.52
22:1n-9	0.66 ± 0.21	0.32–20.45	1.76	0.35	0.97 ± 0.62	0.37 ± 0.17
22:4n-6	25.23 ± 6.34	24.15 ± 5.73	46.47	32.38	26.80 ± 6.69	44.00 ± 2.89
22:5n-6	4.99 ± 1.47	4.01 ± 1.72	13.55	6.55	7.50 ± 2.88	12.54 ± 1.76
22:5n-3	14.42 ± 2.74	16.86 ± 3.26	7.92	14.20	13.24 ± 5.84	12.01 ± 3.05
22:6n-3	41.15 ± 12.14	43.19 ± 10.03	7.07	17.12	12.80 ± 9.55	15.37 ± 2.76
24:0	39.44 ± 7.63	35.11 ± 15.82	38.23	32.90	33.43 ± 10.28	49.58 ± 1.40
24:1n-9	43.01 ± 10.30	27.42–134.75	41.39	36.64	41.08 ± 14.89	32.80 ± 4.12
26:0	2.10 ± 0.54	2.84 ± 1.44	3.81	3.29	2.32 ± 0.84	2.73 ± 0.61
26:1n-9	2.03 ± 0.57	1.13–5.52	2.64	3.06	2.48 ± 1.35	1.24 ± 0.15
Total fatty acids	873.62 ± 110.33	893.35 ± 105.68	964.48	837.19	861.46 ± 103.14	925.67 ± 39.32
24:0/22:0	2.624 ± 0.285	2.901 ± 0.563	3.444	3.723	2.580 ± 0.829	2.453 ± 0.225
24:1n-9/22:0	2.891 ± 0.701	1.481–44.658	3.729	4.147	4.283 ± 4.317	1.612 ± 0.126
26:0/22:0	0.140 ± 0.033	0.238 ± 0.093	0.343	0.372	0.215 ± 0.128	0.136 ± 0.040
26:1n-9/22:0	0.137 ± 0.042	0.051–0.895	0.238	0.346	0.557 ± 1.161	0.062 ± 0.012
22:6n-3/22:5n-3	2.892 ± 0.689	2.758 ± 0.667	0.892	1.206	0.995 ± 0.668	1.326 ± 0.314
22:5n-6/22:4n-6	0.198 ± 0.048	0.180 ± 0.058	0.292	0.202	0.289 ± 0.104	0.285 ± 0.028
20:4n-6/18:2n-6	1.699 ± 0.364	1.797 ± 0.432	5.116	2.129	1.116 ± 0.331	1.414 ± 0.046

[a]Patients with X-ALD that were fed on elemental diets by intubation. Fatty acids are given in picomoles per million of cells. Grouped data are means ± 1 SD. The most characteristic fatty acid ratios that are usually altered in peroxisomal patients are shown in the table. The range, rather than the mean, is given for 22:1, 24:1 and 26:1, and for the ratios of the latter two fatty acids to 22:0 because of the wide variation due to diet (see footnote to Table 1). Abbreviations as in Table 1.

the normal range in red cells and low in plasma. In well-nourished X-ALD/AMN patients, these fatty acids are normal, and in vegetarian healthy children both are high. In confirmation of previous studies (38,39), the present results indicate that, in the developing human, nutrition plays an important role even under normal conditions, and that in normal children, as well as in X-ALD and AMN patients, DHA deficiency may be artificially produced by a low n-3 intake. In normal children, however, brain DHA levels are preserved whereas in PB patients they are drastically decreased (23–25). Similarly, insufficient n-6 PUFA intake may also cause insidious arachidonate deficiency in peroxisomal patients, that unlike n-3 deficiency, does not decrease AA tissue levels. Even so, the possibility of a Δ6 and/or Δ5 desaturase defect as a cause of AA deficiency should be further investigated. Although only seen in plasma, it is well possible that suboptimal levels of arachidonate may play some role in the pathogenesis of the disease. Peroxisomal patients do not grow normally, and a correlation has lately been found between the levels of blood AA and growth in preterm human infants (40).

In view of our findings, we believe that it is important to monitor blood PUFA patterns in patients with disorders of PB to detect DHA deficiency early. When DHA deficiency is seen in red blood cells, it would be advisable to provide DHA orally to possibly normalize brain DHA levels in peroxisomal patients. Although DHA ethyl ester is not yet approved as a drug, early clinical trials (made possible thanks to a grant from the National Institutes of Health, Bethesda, MD) have produced promising results (26); other clinical trials with DHA ethyl ester are underway in Europe. As an n-3/n-6 imbalance may exist in peroxisomal patients, it is necessary to closely monitor AA levels, especially when providing DHA, and to increase the dietary AA intake when necessary. Normalization of all PUFA aberrations in peroxisomal patients, at least in blood, would seem to be

TABLE 3

Plasmalogen Composition of Plasma in Peroxisomal Patients and Controls

Plasmalogen[a]	Ominivore controls 1–58 yr (n = 27)	X-ALD/AMN 5–54 yr (n = 26)	X-ALD 6 yr	X-ALD 12 yr	Generalized peroxisomal 2–19 yr (n = 25)	Vegetarian controls 7–10 yr (n = 4)
16:0DMA	50.14 ± 15.07	43.94 ± 13.72	24.40	23.81	20.52 ± 10.21	42.87 ± 5.39
18:0DMA	42.22 ± 15.43	35.85 ± 15.91	19.48	11.44	18.84 ± 13.69	39.89 ± 3.65
18:1DMA[b]	17.67 ± 5.65	15.92 ± 5.43	2.04	4.36	6.24 ± 4.85	9.84 ± 1.93
Total plasmalogen	113.8 ± 36.0	99.6 ± 33.3	47.8	41.3	47.1 ± 26.8	94.5 ± 7.9
16:0DMA/16:0	0.020 ± 0.006	0.015 ± 0.008	0.006	0.009	0.009 ± 0.005	0.019 ± 0.006
18:0DMA/18:0	0.045 ± 0.014	0.035 ± 0.018	0.014	0.009	0.025 ± 0.014	0.046 ± 0.008
18:1DMA/18:1	0.007 ± 0.003	0.005 ± 0.003	0.000	0.001	0.003 ± 0.002	0.006 ± 0.004

[a]The individual dimethyl acetal (DMA) of the major alkenyl groups (16:0DMA, 18:0DMA, and the two isomeric forms of 18:1DMA) were added to obtain the total. The ratios of each plasmalogen DMA to the corresponding fatty acid methyl esters are of diagnostic value in peroxisomal disorders and are given in the table. Plasmalogen figures represent nanomoles per milliliter of plasma. Abbreviations as in Table 1.

[b]Only the major isomer oleyl aldehyde is reported here.

TABLE 4

Plasmalogen Composition of Erythrocytes in Peroxisomal Patients and Controls[a]

Plasmalogen	Omnivore controls 4–58 yr (n = 33)	X-ALD/AMN 5–54 yr (n = 26)	X-ALD 6 yr	X-ALD 12 yr	PB disorders 2–19 yr (n = 25)	Vegetarian controls 5–9 yr (n = 4)
16DMA	16.68 ± 2.16	14.93 ± 3.09	14.91	12.76	12.19 ± 2.96	16.52 ± 0.76
18:0DMA	29.87 ± 3.06	26.10 ± 5.12	23.33	20.46	20.17 ± 5.05	28.93 ± 2.15
18:1DMA	8.11 ± 1.51	8.60 ± 2.62	4.01	5.70	5.32 ± 2.48	7.28 ± 0.77
Total plasmalogen	55.7 ± 6.4	51.2 ± 7.1	43.4	40.1	37.6 ± 10.9	53.8 ± 2.7
16:0DMA/16	0.094 ± 0.014	0.085 ± 0.018	0.069	0.075	0.063 ± 0.016	0.092 ± 0.008
18:0DMA/18	0.214 ± 0.022	0.194 ± 0.028	0.149	0.134	0.156 ± 0.035	0.202 ± 0.007
18:1DMA/18:1	0.075 ± 0.016	0.076 ± 0.016	0.027	0.057	0.051 ± 0.015	0.075 ± 0.010

[a]Plasmalogen figures are given in picomoles per million of red cells; (see Table 3 for additional explanations and abbreviation). See Table 1 for other abbreviation.

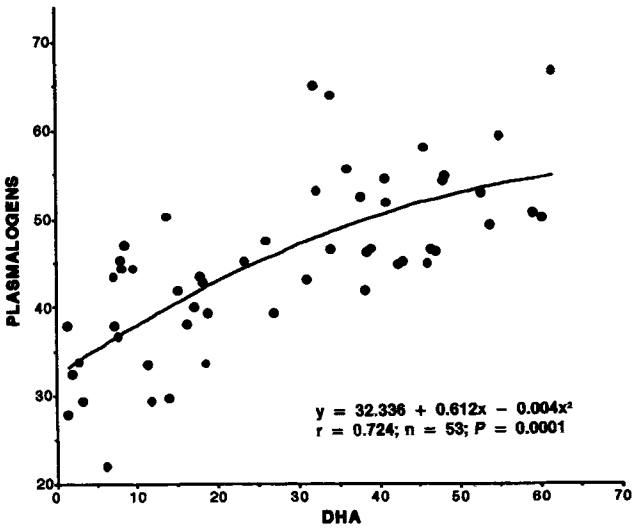

FIG. 3. Total plasmalogens, as measured by gas chromatography of the dimethyl acetals derived from the major aldehydogenic chains (16:0, 18:0 and 18:1), plotted against DHA concentration in red blood cells of patients with peroxisomal disorders. All cases (but not normal controls) are included in this graph, i.e., X-ALD/AMN as well as PB patients (n = 53). Values are pmol/10^6 cells. The parabolic regression line obtained is statistically very significant. See Figure 1 for abbreviations.

an important first step in clarifying the pathogenesis of peroxisomal disorders.

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Differential and Interactive Effects of Calcium Channel Blockers and Cholesterol Content of the Diet on Jejunal Uptake of Lipids in Rabbits

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The present study was undertaken to determine the effects of two classes of calcium channel blockers (CCB), nisoldipine (N) and verapamil (V), on the jejunal uptake of lipids in rabbits. The uptake of cholesterol and long-chain fatty acids into rabbit jejunum was examined after 6 and 36 min of exposure to N or V *in vitro* ("acute" studies), and after 3-wk feeding of N or V ("chronic" studies). Animals were fed either a low (0.08%) cholesterol diet (LCD) or a high (2.8%) cholesterol diet (HCD), with or without N or V added. Acute *in vitro* exposure of the jejunum to N or V did not affect the uptake of cholesterol or palmitic acid in rabbits fed LCD or HCD. The effect of N or V feeding depended upon the cholesterol content of the diet; adding N or V to LCD increased cholesterol uptake while adding N or V to HCD enhanced or lowered cholesterol uptake, respectively. Both N and V increased the uptake of stearic acid in LCD. N in HCD had no effect on fatty acid uptake, whereas V lowered the uptake of stearic and linoleic acids and increased the uptake of oleic acid. These changes in lipid uptake were not due to variation in the animals' food intake, body weight gain, or intestinal mucosal surface area. The chronic administration of N or V results in an intestinal adaptive process that alters the jejunal uptake of lipids, the direction of which is influenced by the class of CCB, and by the cholesterol content of the diet. The serum lipid-lowering effect of administering N to rabbits fed HCD demonstrated previously is unlikely to be the result of a decrease in intestinal lipid uptake.
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Pharmacologic agents that block calcium entry into cells can be chronically administered for the treatment of several disorders, including angina pectoris, hypertension, supraventricular tachycardia and posthemorrhagic cerebral vasospasm (1). In addition, numerous accounts of the potential antiatherogenic effects of these compounds, collectively referred to as calcium channel blockers (CCB), have been published (2–9). Administering CCB to animals fed a high cholesterol diet (HCD) reduces the formation of atherosclerotic lesions by a mechanism that is still unclear. Some, but not all (10–13), studies in humans also suggest a favorable influence of CCB on atherosclerosis. The mechanisms of this action are unknown (14–17).

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; CCB, calcium channel blockers; HCD, high cholesterol diet; Isc, short circuit current; LCD, low cholesterol diet; N, nisoldipine; TDC, taurodeoxycholic acid; UWL, unstirred water layer; V, verapamil.

In our previous study using a rabbit model of atherosclerosis, serum total cholesterol levels were reduced by 23–44% in animals administered the CCB nisoldipine (N; oral dose 1 mg/kg), and aortic cholesterol content was also significantly diminished by feeding N (9). Reductions in serum cholesterol levels have been reported by others (5,8,18,19).

Recently, radioligand binding and short circuit current (Isc) studies have suggested the presence of voltage-dependent calcium channels in rabbit ileum (20); the magnitude of the effects may vary with different classes of CCB. The effect of chronic intestinal CCB exposure on lipid uptake processes in the small intestine has not been reported previously. The present study was undertaken to determine whether *in vitro* jejunal lipid uptake is affected by the administration of two different classes of CCB, a 1,4-dihydropyridine, N, and a phenylalkylamine, verapamil (V). The results suggest that a modified intestinal uptake of lipids due to CCB does not represent the mechanism by which N reduces serum and tissue lipid levels in the rabbit.

MATERIALS AND METHODS

Animals. Sixty male New Zealand white rabbits (8–10 wk old, 1.5–2.0 kg) were used in the study. All animals were maintained on Baby Rabbit Pellets (United Feeds, Edmonton, Alberta, Canada) for 1 wk before being assigned randomly to control or to experimental groups. The rabbits were housed individually under identical conditions with controlled lighting and ventilation. Food intake and body weight were monitored, and water was provided *ad libitum*. Blood samples were collected from the marginal ear vein for determination of serum cholesterol, triglyceride and glucose concentrations at the initiation and completion of the treatment period.

Study design. The study was designed to determine whether jejunal uptake *in vitro* was affected by 6 and 36 min *in vitro* ("acute" exposure) and 3-wk feeding ("chronic" exposure) to the dihydropyridine derivative, N, and to the phenylalkylamine, V. Acute effects of N exposure on the jejunal uptake of cholesterol and palmitic acid (16:0) were determined. Chronic effects of N or V on the uptake of cholesterol, stearic acid, oleic acid and linoleic acid were also followed.

Acute experiments. In the acute experiments, one group of twelve animals was maintained on Purina Chow (hereafter referred to as low cholesterol diet or LCD) and a second group of twelve rabbits was fed the same pellets modified to contain 2.8% cholesterol (w/w) (referred to as HCD). The HCD was prepared by adding cholesterol (Sigma Chemical Co., St. Louis, MO) to the

low cholesterol pelleted chow diet. Briefly, 4 kg allotments of chow were thoroughly mixed with 100 g of cholesterol dissolved in 500 mL of diethyl ether (reagent grade; Fisher Scientific Ltd., Fair Lawn, NJ). The prepared food mixture was then spread out on trays and placed in a fume hood to dry for 48 h. The cholesterol content of eight random duplicate samples of the prepared diet was estimated, using a modified Folch's lipid extraction procedure (21,22) and a commercial enzymatic colorimetric test for cholesterol determination (Boehringer Mannheim, Mannheim, Germany), in which the organic layer was reconstituted in isopropanol. The cholesterol content of HCD was 2.8 ± 0.2 (mean ± SEM), and the cholesterol content of LCD was 0.8% (8 mg/kg).

Chronic experiments. For the chronic drug feeding experiments, animals were divided into six groups: LCD; HCD; LCD + N; LCD + V; HCD + N and HCD + V. There were six animals in each of these six groups.

Drug doses and administration. Animals were treated for a period of 3 wk. Solubilized N and V (as described below) were provided orally by syringe feeding at daily doses of 1 mg/kg body weight and 4 mg/kg, respectively. The dose of N was selected to duplicate the model of Senaratne *et al.* (9) and to avoid hemodynamic effects of the drug. A dose of 1 mg/kg N effectively reduces atherosclerosis and is also well below the dose of 20 mg/d, which was previously shown to decrease mean arterial pressure in cholesterol-fed New Zealand white rabbits (6). The V dose of 4 mg/kg was selected to approximate clinical doses used in humans (240–360 mg/d on average for a 70 kg body weight adult) (23), rather than the higher doses used in most animal studies reported (3,4). This dose is also not associated with hemodynamic effects (4).

In preliminary studies, the introduction of aqueous solution by a syringe into the mouth was shown not to influence the effect of cholesterol feeding (Kappagoda, C.T., unpublished observations). Thus, a control vehicle was not used in the present study.

Chemicals. Unlabelled cholesterol and fatty acids were all >99% pure as supplied by Sigma Chemical Co. Taurodeoxycholic acid (TDC) was obtained from Calbiochem Corp. (La Jolla, CA). To measure the intestinal adherent mucosal fluid volume, [^{3}H]inulin (molecular weight approximately 5000) was cited, which was obtained from Amersham Canada Ltd. (Oakville, Canada). The [1-^{14}C]-labelled cholesterol was also obtained from Amersham Canada Ltd., and [1-^{14}C]-labelled palmitic acid, stearic acid, oleic acid and linoleic acid were obtained from New England Nuclear (Boston, MA). All other compounds used (Fisher Scientific Ltd.) were of reagent grade.

N in powdered form (Miles Pharmaceutical Ltd., New Haven, CT) was solubilized in 3.5 mL of 95% ethanol (<1.5% of the final administration volume) and distilled deionized water for oral administration. V (Sigma Chemical Co.) was solubilized in distilled deionized water. The drugs were used at a concentration so that the average quantity of fluid provided daily to achieve the required dose was 0.35 mL. The mean quantity of ethanol received each day by the N administered rabbits was 5.4 µL, which is well below the chronic ethanol levels (approximately 26 mL/d for 6–7 wk) associated with altered uptake of lipids and glucose in rabbit jejunum (24).

Preparation of incubation solutions. Test solutions containing cholesterol or fatty acids were prepared as described previously (25). All fatty acid concentrations in the micellar solutions were 0.1 mM in 20 mM TDC. Cholesterol concentrations of 0.1 mM in both 10 and 20 mM TDC were used. For the acute studies, test solutions were prepared with the addition of N in concentrations ranging from 10^{-8}M to 10^{-4}M. All preparation and experimentation with N was conducted in a darkened room to avoid possible photodegradation of the drug.

Tissue preparation. The rabbits were anesthetized with pentobarbitone sodium (25 mg/kg), and the jejunal segment was quickly removed and rinsed with 150 mL of ice cold saline. A previously-validated method for determining *in vitro* nutrient uptake was used (26). For acute exposure experiments, half of the tissues were preincubated in Krebs-bicarbonate containing N ranging from 10^{-8}M to 10^{-4}M for 30 min while the other half was preincubated in the drug-free Krebs solution for the same time period. The rate of lipid uptake was expressed as nmol of probe taken up into the mucosa per 100 mg dry weight of mucosa (nmol/100 mg mucosa · min^{-1}). The weight of the mucosa as well as the ratio of mucosa/submucosa was determined by scraping the mucosal layer from the underlying layer with a glass slide. Uptake rates were also calculated as nmol of probe taken up per minute per unit serosal surface area.

The morphology of the jejunal tissue was assessed as previously described (27–29).

The values obtained for the different groups of animals are reported as the mean ± SEM of the results from 6–12 animals in each group.

Serum cholesterol and triglyceride determinations. The cholesterol, triglyceride and glucose measurements in the serum were made by use of an automated system (Multistat III; Instrumentation Laboratories, Lexington, KY), which incorporated the methods of Allain *et al.* (30) and Pinter *et al.* (31).

RESULTS

Animal characteristics. The oral administrations of N and V were well tolerated, and there were no deaths or significant adverse events during the 3-wk course of the study. All rabbits consumed the same amount of food, with the exception of the higher food intake in animals fed LCD + N as compared with LCD, yet body weight gain was equal among all groups (Tables 1–3).

As expected, serum cholesterol and triglyceride concentrations were higher in rabbits fed HCD as compared with those fed LCD (Table 1). Adding V to LCD (LCD + V) or HCD (HCD + V) did not significantly affect the lipid levels (Table 2). However, rabbits fed HCD + N (Table 2) had lower serum cholesterol levels (880.6 ± 93.6 mg/dL in animals fed HCD vs. 587.5 ± 22.6 mg/dL in the HCD + N group). Although serum triglyceride levels were lower in animals fed HCD + V vs. those fed

TABLE 1

Characteristics of Animals Used in the Study of Acute Drug Exposure[a]

	LCD	HCD
Weight gain	0.60 ± 0.05	0.54 ± 0.04
Total food consumed (g/3 wk)	2313 ± 172	2364 ± 154
Cholesterol intake (g/3 wk)	18.5 ± 1.4	59.10 ± 3.84
Serum parameters (mg/dL)		
Cholesterol (0)	85.4 ± 5.0	79.7 ± 5.6
Cholesterol (3)	77.9 ± 10.7	841.5 ± 132.5[b]
Triglyceride (0)	80.0 ± 7.5	109.3 ± 16.4
Triglyceride (3)	81.2 ± 13.5	132.6 ± 16.9
Glucose (0)	161.0 ± 21.6	175.1 ± 31.4
Glucose (3)	112.7 ± 16.2	135.6 ± 7.0

[a]Values are means ± SE; LCD, low cholesterol diet; HCD, high cholesterol diet; (0), value at initiation of study; (3), value after 3-wk feeding.

[b]$P < 0.05$ LCD vs. HCD.

HCD (Table 3), this difference was not statistically significant. Serum glucose levels were similar in animals fed LCD or HCD with or without N or V (Tables 2 and 3).

Intestinal morphology. Adding N or V to HCD lowered the dry weight of the jejunum (HCD + N or HCD + V vs. HCD), but adding a CCB to LCD did not affect the weight of the jejunum (Table 4). Because of these differences in jejunal weight, and the lower percentage of the intestinal wall comprised of mucosa in animals fed HCD + V, rates of uptake were expressed on the basis of the dry weight of the mucosa rather than the dry weight of the entire wall of the jejunum.

The jejunal villus height was significantly increased in HCD + N and HCD + V groups as compared to HCD animals not given CCB, but because of adjustments in the dimensions of the width and thickness of the villi, the mucosal surface area was not altered among any of the groups.

Lipid uptake: acute studies. The jejunal uptake of 0.1 mM cholesterol and 0.1 mM palmitic acid (16:0) in 20 mM TDC was unaffected by 6 and 36 min *in vitro* exposure to increasing concentrations (10^{-8}–10^{-4}M) of N (data not shown). No change was observed in rabbits fed either LCD or HCD. The effect of V on the *in vitro* uptake of lipids in animals fed LCD or HCD was not studied.

Lipid uptake: chronic studies. The effect of feeding of N for 3 wk on the jejunal uptake of cholesterol depended upon the ratio of TDC/cholesterol in the test solution (Table 5). When 10 mM TDC was used to solubilize 0.1 mM cholesterol, uptake was increased in both LCD + N and HCD + N groups, as compared to the LCD or HCD groups. In contrast, when 20 mM TDC was used to solubilize cholesterol, cholesterol uptake was reduced in the LCD + N group as compared to that in the LCD group. By comparison, when V was added to LCD, cholesterol uptake increased compared to that in the LCD group, while feeding HCD + V decreased cholesterol uptake compared to HCD alone (Table 6). Thus, adding N or V to LCD increased cholesterol uptake, while V + HCD lowered cholesterol uptake and N + HCD enhanced uptake. Both N and V increased the jejunal uptake of stearic acid (18:0) in LCD. N in HCD had no effect on fatty acid uptake compared to HCD alone, whereas V lowered the uptake of stearic and linoleic acids yet increased the uptake of oleic acid (Tables 5 and 6).

DISCUSSION

Lipid uptake into the jejunum is differentially affected by the two classes of CCB. In addition, the direction of these effects is influenced by the level of cholesterol in the diet. The effects of N or V are likely due to an adaptive process in the intestine occurring over the 3 wk of feeding, because N did not affect cholesterol or palmitic acid uptake when added directly to the incubation medium.

Interestingly, the ratio of cholesterol/TDC used to solubilize cholesterol affected the direction of uptake response in animals administered N but not V. Increasing the ratio of TDC/cholesterol has previously been shown to lower *in vitro* cholesterol uptake, possibly due to reduced partitioning from the micelle to the monomeric phase adjacent to the brush border membrane (25,32). The reduction in cholesterol uptake by N + LCD when

TABLE 2

Characteristics of Nisoldipine (N) Administered and Control Animals[a]

	LCD	LCD + N	HCD	HCD + N
Weight gain (kg/3 wk)	0.64 ± 0.03	0.63 ± 0.05	0.64 ± 0.05	0.57 ± 0.05
Total food consumed (g/3 wk)	1614 ± 54	1917 ± 119[b]	1977 ± 104	1805 ± 106
Cholesterol intake (g/3 wk)	12.9 ± 0.4	15.3 ± 1.0	49.41 ± 2.58	45.09 ± 2.65
Serum parameters (mg/dL)				
Cholesterol (0)	103.4 ± 9.5	107.3 ± 6.0	108.9 ± 9.6	105.3 ± 5.8
Cholesterol (3)	73.1 ± 4.1	63.9 ± 3.3	880.6 ± 93.6[c]	587.5 ± 22.6[d]
Triglyceride (0)	169.9 ± 53.7	123.0 ± 24.8	156.4 ± 24.9	137.0 ± 21.5
Triglyceride (3)	91.4 ± 14.1	87.1 ± 9.4	126.7 ± 15.8	139.8 ± 31.9
Glucose (0)	154.7 ± 9.4	165.4 ± 7.6	146.8 ± 5.1	153.4 ± 4.9
Glucose (3)	161.6 ± 12.5	147.1 ± 14.1	144.7 ± 6.1	139.6 ± 8.1

[a]LCD + N, low cholesterol diet plus nisoldipine; HCD + N, high cholesterol diet plus nisoldipine. See footnote a in Table 1 for other abbreviations and details. [b]$P < 0.05$ LCD vs. LCD + N. [c]$P < 0.05$ HCD, HCD + N vs. LCD, LCD + N. [d]$P < 0.005$ HCD vs. HCD + N.

TABLE 3

Characteristics of Verapamil (V) Administered and Control Animals[a]

	LCD	LCD + V	HCD	HCD + V
Weight gain (kg/3 wk)	0.72 ± 0.04	0.64 ± 0.06	0.65 ± 0.09	0.60 ± 0.08
Total food consumed (g/3 wk)	2421 ± 153	1946 ± 77	2287 ± 71	2427 ± 254
Cholesterol intake (g/3 wk)	19.4 ± 1.2	15.6 ± 0.6	57.15 ± 1.76	59.82 ± 7.04
Serum parameters (mg/dL)				
Cholesterol (0)	62.4 ± 3.9	100.4 ± 9.3	103.1 ± 5.9	71.5 ± 8.1
Cholesterol (3)	57.3 ± 5.9	70.4 ± 6.2	1010.6 ± 295.2[b]	992.1 ± 217.8
Triglyceride (0)	91.9 ± 18.1	121.2 ± 27.8	132.1 ± 27.3	95.1 ± 15.1
Triglyceride (3)	73.4 ± 16.1	56.2 ± 15.5	156.6 ± 49.9[b]	108.6 ± 27.2[b]
Glucose (0)	147.0 ± 11.2	139.5 ± 5.4	213.3 ± 37.8	191.8 ± 29.1
Glucose (3)	154.3 ± 8.9	145.8 ± 10.3	156.2 ± 5.4	155.8 ± 16.7

[a]LCD + V, low cholesterol diet plus verapamil; HCD + V, high cholesterol diet plus verapamil. See footnote a in Table 1 for other abbreviations and details. [b]$P < 0.05$ HCD, HCD + V vs. LCD, LCD + V.

solubilized in 20 mM TDC and the enhancement of cholesterol uptake by N + LCD with 10 mM of TDC (Table 5) may have been due to the effect of N on the partitioning of cholesterol from the TDC micelle. This possibility is strengthened by the observation of a variable effect of N in HCD on cholesterol uptake. The directionally similar effects on cholesterol uptake of V in LCD and HCD with 10 and 20 mM TDC may be due to greater intestinal binding of V (20), or to variable degrees of tissue specificity among the CCB (33). In this study, we did not assess the effect of N or V on intestinal uptake of calcium, so we are not able to say whether the influence of CCB on the intestine morphology and function is actually the result of a change in Ca^{2+} flux.

A similar pattern was identified for the uptake of stearic acid; an enhanced uptake of 18:0 was observed in animals fed N or V while on the LCD, but a reduced uptake was found in animals given V while on the HCD. However, not all of the fatty acids behaved in the same manner (Tables 5 and 6). Possible heterogeneity of the uptake of lipids has been suggested based on theoretical considerations (34), and the variable effect of N or V on the mono- or polyunsaturated as compared with the saturated fatty acids would support this view. In addition, however, there were qualitative differences in the effect of N or V on the uptake of fatty acids. Thus, although the mechanisms of the effect of CCB lipid uptake have not been established in this study, the mechanisms appear to vary between these two different classes of drugs.

The differential effects of N and V on the intestine were particularly apparent in the animals fed HCD, and this differential effect has been noted in other tissues. In cholesterol-enriched but not in control-perfused rabbit aorta, V and diltiazem have been shown to antagonize the effect of norepinephrine-stimulated calcium influx (35), and it was suggested that cholesterol enrichment of the plasma membrane may have exposed sites that were otherwise unavailable for binding to the CCB. In our

TABLE 4

Jejunal Characteristics and Morphology of Study Animals[a]

	LCD	HCD	LCD + N	HCD + N	LCD + V	HCD + V
Dry weight mg/unit serosal surface area	35.7 ± 2.3	39.2 ± 1.5	31.6 ± 1.2	31.7 ± 1.8[b]	34.1 ± 1.3	32.5 ± 1.6[c]
% of intestinal wall comprised of mucosa	84.1 ± 1.8	83.6 ± 1.1	78.4 ± 3.0	82.9 ± 1.9	78.9 ± 2.1	72.2 ± 1.2[c]
Crypt depth (μm)	50 ± 2.9	43 ± 2.8	51 ± 2.6	69 ± 3.0[b]	44 ± 2.7	44 ± 2.1
Villus height (μm)	603 ± 29	599 ± 24	573 ± 25	717 ± 33[b]	680 ± 42	752 ± 50[c]
Villus width at half height (μm)	94 ± 4	126 ± 6	98 ± 4	158 ± 8	114 ± 6	124 ± 6
Villus bottom width (μm)	114 ± 7	135 ± 5	122 ± 5	166 ± 6	127 ± 8	134 ± 7
Villus thickness (μm)	431 ± 28	210 ± 12	327 ± 30	390 ± 50	340 ± 25	267 ± 11
Villus surface area (μm^2/villus)	666 ± 32	428 ± 18	511 ± 23	846 ± 45	651 ± 37	622 ± 47
Number of villi/mm^2 serosa	21.3 ± 1.4	35.6 ± 1.2	25.3 ± 1.0[d]	15.7 ± 0.6[b]	24.2 ± 1.7	28.8 ± 1.5
Mucosal surface area (mm^2/mm^2 serosa)	14.0 ± 1.0	15.2 ± 0.9	13.0 ± 0.9	13.2 ± 0.8	15.81 ± 1.5	17.8 ± 1.3

[a]See footnote a in Tables 2 and 3 for abbreviations and details. [b]$P < 0.05$ HCD vs. HCD + N. [c]$P < 0.05$ HCD vs. HCD + V. [d]$P < 0.05$ LCD vs. LCD + N.

TABLE 5

Effect of Chronic Oral N Intake (1 mg/kg • d^{-1}) on Jejunal Uptake of Cholesterol and Fatty Acid[a]

Substrate	LCD	LCD + N	HCD	HCD + N
Cholesterol[1]	0.16 ± 0.03	0.08 ± 0.02[b]	0.13 ± 0.02	0.10 ± 0.01
Cholesterol[2]	0.30 ± 0.06	1.01 ± 0.10[b]	0.13 ± 0.02	1.31 ± 0.13[c]
Stearic acid (18:0)	0.30 ± 0.04	0.56 ± 0.11[b]	0.30 ± 0.04	0.46 ± 0.04
Oleic acid (18:1)	0.30 ± 0.04	0.21 ± 0.05	0.24 ± 0.05	0.29 ± 0.05
Linoleic acid (18:2)	0.21 ± 0.03	0.32 ± 0.06	0.42 ± 0.10	0.59 ± 0.08

[a]See Table 2, footnote a, for details and abbreviations. The concentration of cholesterol was either (0.1 mM) in 20 mM taurodeoxycholic acid (cholesterol[1]) or 0.1 mM in 10 mM taurodeoxycholic acid (cholesterol[2]). The concentration of the fatty acids was 0.1 mM in 20 mM taurodeoxycholic acid. The rate of uptake was expressed as nmol/100 mg mucosa • min^{-1}. [b]$P < 0.05$ LCD vs. LCD + N. [c]$P < 0.05$ HCD vs. HCD + N.

cholesterol-fed model, alterations in enterocyte membrane lipid composition achieved by feeding cholesterol (HCD vs. LCD) (36) may affect the binding site for V differently than that of N because different CCB appear to bind to different sites on calcium channel proteins (37).

Several possible mechanisms of intestinal adaptation to various stimuli have been suggested (38,39), including changes in the effective resistance of the unstirred water layer (UWL) or the lipid composition of the brush border membrane (28,29,38,40,41). The effective resistance of the UWL has been reported to decrease in animals fed high-cholesterol diets, and this would be expected to be associated with a higher uptake of lipids (42). In this study, we did not assess the effective resistance of the UWL. However, we did observe a reduced uptake of most lipids in the HCD + V group and no reductions in the HCD + N group (Tables 5 and 6). Thus, it is unlikely that the effect of N or V on cholesterol or fatty acid uptake was due to an effect on UWL resistance. The movements of lipids through the intestinal brush border membrane is believed to be mainly a passive process, although a carrier-mediated component has been suggested for fatty acids and possibly cholesterol (43). The *in vitro* CCB have been associated with the *de novo* synthesis of LDL receptors, suggesting their potential for synthetic activity of membrane-bound proteins (16). We have not assessed the effect of N or V on brush border membrane lipid composition or membrane fatty acid binding protein, so it remains unknown whether the altered membrane permeability might have been due to an effect of N or V on enterocyte lipid metabolism and brush border membrane lipid composition.

In spite of a significant increase in villus height in both the HCD + N and the HCD + V animals, no variations in the mucosal surface area were observed between drug-administered vs. control groups (Table 4). Two-dimensional measurements (villus height and width) of mucosal surface area do not necessarily provide reliable assessments of surface area. For example, in this study the villus density (number of villi/mm^2 serosa) decreased in animals fed HCD + N and HCD + V, countering the increase in villus height, so that the mucosal surface area was unchanged. Thus, the effect of feeding N and V with LCD or HCD cannot be explained on the basis of variations in the intestinal mucosal surface area, nor can the results be explained on the basis of any difference in the animals' body weight gain or food intake (Tables 1–4).

Effects of CCB on intracellular lipid metabolizing enzymes (such as fatty acid desaturase, acylation and deacylation enzymes, or the cholesterol and phospholipid metabolizing enzymes) could alter brush border membrane lipid composition, thereby resulting in altered permeability properties of the membrane (44). A calcium, calmodulin-dependent kinase has been suggested to play a role in the short-term regulation (*via* phosphorylation and concomitant inactivation) of HMG–CoA reductase, the rate-limiting enzyme for cholesterol synthesis (45). Reduced calcium flux might upregulate the expressed activity HMG–CoA reductase by promoting the dephosphorylation of the enzyme. However, if a calcium-dependent step was affected by the presence of

TABLE 6

Effect of Chronic Oral V Intake (4 mg/kg • day^{-1}) on Jejunal Uptake of Cholesterol and Fatty Acid[a]

Substrate	LCD	LCD + V	HCD	HCD + V
Cholesterol[1]	0.35 ± 0.03	0.94 ± 0.11	0.97 ± 0.12	0.36 ± 0.04[c]
Cholesterol[2]	0.53 ± 0.05	1.08 ± 0.15[b]	0.95 ± 0.07	0.41 ± 0.04[c]
Stearic acid (19:0)	0.89 ± 0.07	1.29 ± 0.11[b]	1.60 ± 0.11	0.99 ± 0.05[c]
Oleic acid (18:1)	1.41 ± 0.17	1.01 ± 0.09	1.65 ± 0.11	2.43 ± 0.29[c]
Linoleic acid (18:2)	1.21 ± 0.12	1.12 ± 0.10	1.95 ± 0.21	1.45 ± 0.12[c]

[a]See Table 3, footnote a, and Table 5, footnote a, for details and abbreviations. [b]$P < 0.05$ LCD vs. LCD + V. [c]$P < 0.05$ HCD vs. HCD + V.

CCB in the enterocyte, both classes of drug would be expected to affect the intracellular synthesis of cholesterol and thereby would have directionally similar effects on the brush border membrane composition of cholesterol. In rat brush border membrane vesicles intraluminal calcium decreased the fluidity by alterations in membrane sphingomyelin content by unknown, presumably indirect, mechanisms (46). In cultured macrophages, CCB inhibit cholesterol esterification (47). The mechanism for this has not yet been defined, but it is apparently independent of the calcium-blockade effect, and could involve a direct effect on the acyl coenzyme A:cholesterol acyltransferase (ACAT) reaction in this tissue. Furthermore, not all classes of CCB showed this inhibitory effect. It is unknown whether a similar effect might apply to the intestine, but clearly alterations in the activity of ACAT also influence the intestinal uptake of cholesterol (48). The upregulated state of ACAT following cholesterol feeding as well as possible differential effects of CCB on this enzyme could account for some of the variable effects noted between CCB and diet effects.

CCB affect Isc measured across the ileal tissue, with V having a quantitatively greater effect than other classes of CCB (20). This change in Isc represents the net effect of calcium channel blockade on the flux of anions and cations from the mucosa to the serosa, and from the serosa to the mucosa. In the presence of CCB, equally enhanced mucosal-to-serosal movement of sodium and chloride exceed serosal-to-mucosal movement of these ions. Residual fluxes (likely bicarbonate ions) and Isc are decreased (49). Removal of sodium from the bulk phase reduces the uptake of fatty acids (43,50), but it is unknown what effect an enhanced mucosal-to-serosal flux of sodium might have on lipid uptake. In cardiac myocytes and peritoneal mast cells, reductions in extracellular calcium increase sodium influx and permeability; these effects are associated with an enhancement of sodium pump (Na^+/K^+-ATPase) activity (51). It is unknown whether calcium plays a role in the physiological control of the intestinal basolateral membrane sodium pump, or whether CCB exert an important effect. There appear to be at least two isoforms of this ATPase in the intestine (52), which could also be differentially affected by cholesterol feeding or by different classes of CCB. CCB in the serosal medium bathing jejunal tissue reduce the magnitude of glucose stimulated Isc in LDC but not in HCD animals (Hyson, D.A., Thomson, A.B.R., Fedorak, A.N., and Kappagoda, C.T., in preparation). However, the variable effect of V on the uptake of the three fatty acids examined in this study suggest that the mechanism cannot likely be explained solely on the basis of altered effects of sodium gradients across the enterocyte.

Studies in rabbit ileal brush border (53) and chicken enterocytes (54) have suggested a role for calcium in regulating the activity of the intestinal Na/H^+ antiporter. Protein kinase C and cAMP mediated inhibition of the activity of the Na/H^+ antiporter was associated with increased cell surface pH in rat jejunal villus cells (55). Although calcium ionophores did not cause a similar inhibition, it is unknown whether altered intracellular calcium levels were achieved; thus, a role for calcium was not ruled out. It is unknown if CCB would enhance Na/H^+ exchange. However, it would be expected that increasing the activity of the antiporter would increase the pH of the acidic microclimate adjacent to the mucosal membrane (56). Since fatty acids would be protonated at a low pH, their solubility in micelles would be reduced, and partitioning into the lipid membrane would be enhanced (57). In this study, both N and V enhanced stearic acid uptake in animals fed LCD, and V increased the uptake of oleic acid in animals given HCD.

Our initial interest of the possible effect of CCB on intestinal lipid uptake was stimulated by the observation of the cholesterol-lowering effects (both in serum and aorta) in animals fed N simultaneously with an HCD (9). This cholesterol-lowering effect of N was clearly not due to a decline in the intestinal uptake of lipids (Table 5). While V reduced the uptake of lipids in animals fed HCD (Table 6), it did not reduce levels of cholesterol in serum (Table 3). Thus, it is likely that the effect of N on cholesterol concentration lies beyond the intestine.

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Δ^{22}-β-Muricholic Acid in Monoassociated Rats and Conventional Rats

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Bile acids were analyzed in the bile, small and large intestines, and feces of germ-free rats after a single inoculation with one of six intestinal bacteria that had been originally isolated from human feces. *Bacteroides vulgatus* and *Bifidobacterium longum* preferentially deconjugated tauro-β-muricholic acid and taurocholic acid, respectively. *Clostridium ramosum, Peptostreptococcus productus* and *Lactobacillus gasseri* deconjugated both bile acids, but *Escherichia coli* did not deconjugate either one. Rats inoculated with bacteria that deconjugated tauro-β-muricholic acid produced Δ^{22}-β-muricholic acid in the feces. In contrast, Δ^{22}-cholic acid could not be detected in rats inoculated with bacteria that deconjugated taurocholic acid.
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Intestinal bacteria are known to influence bile acid metabolism (1–3). Deconjugation and desulfation of bile acids have been demonstrated in rats inoculated with a single strain of bacteria (4–6); 7-dehydroxylation, oxidation–reduction and epimerization of bile acids in rats have also been reported (7,8). Recently, Eyssen *et al.* (9) suggested that the side chain of β-muricholic acid might be desaturated in the feces of gnotobiotic rats after a single inoculation with *Eubacterium lentum* that had been isolated from the ceca of conventional rats. Subsequently, this group of investigators also reported the formation of Δ^{22}-β-muricholic acid in the feces of rats after inoculation with *Clostridium* sp. Cl_8 obtained from conventional rats (10).

In the present study we show that desaturation of the side chain of β-muricholic acid (Δ^{22}-β-muricholic acid) occurs upon a single inoculation with predominant bacteria obtained from human feces.

MATERIALS AND METHODS

Determination of tauro-Δ^{22}-β-muricholic acid. Bile acids in bile obtained from conventional rats and germ-free rats were analyzed by high-performance liquid chromatography (HPLC). For this purpose, a Cosmosil $5C_{18}$ column (15 cm × 4.6 mm i.d.; Nakarai Kagaku Co., Kyoto, Japan) was used, and 0.3% ammonium phosphate (pH 7.0)/acetonitrile (20:7, vol/vol) served as mobile phase; the eluent was monitored at 210 nm.

The peak with a relative retention time of 0.36 (peak A) relative to taurocholic acid was detected in bile of conventional rats, while it could not be detected in bile of germ-free rats.

Fraction A was isolated directly from bile of conventional rats by preparative HPLC using a TSK-gel ODS-120T column (25 cm × 2 cm i.d.; Tosoh, Tokyo, Japan) and 0.3% ammonium phosphate (pH 7.0)/acetonitrile (20:8, vol/vol) as mobile phase (monitored at 220 nm) (11). The structure of the compound representing peak A was determined by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectrometry.

^1H NMR spectra were recorded on a Varian XL-400 instrument (Varian, Palo Alto, CA) at 399.950 MHz and ^{13}C NMR spectra on a Varian XL-200 instrument at 50.309 MHz using [$^2H_5$]pyridine as solvent and tetramethylsilane as internal standard (δ_HO and δ_CO). The accuracies of δ_H, J and δ_C were ±0.002 ppm, ±0.3 Hz and ±0.03 ppm, respectively. 2D INADEQUATE (incredible natural abundance double-quantum transfer experiment) ^{13}C NMR spectra were taken with the Varian XL-400 at 100.571 MHz using the CCC2DQ pulse sequence (12,13) of the standard Varian software. The concentration of β-muricholic acid was 498 mg in 1.8 mL of [$^2H_5$]pyridine using a 10-mm sample tube. Fourier transform (FT) conditions were as follows: spectral width, 7936.5 Hz; acquisition time, 0.258 s; pulse delay, 1.5 s; number of transients, 1280; number of free induction decays, 96; conversion time (τ), 6.94 milliseconds (J = 36 Hz). Total experiment time was 60 h. An FT experiment was performed on the 4096 × 2048 matrix with zero-filling in the first domain, and with line-broadening weighting of 40 and 5 Hz, in the first and in the second domain, respectively.

Animals and treatments. Germ-free Wistar male rats (*ca.* 4 months old) bred at Shionogi Aburahi Laboratories (Shiga, Japan) had been housed under germ-free conditions and were monoassociated orally with six strains of bacteria, i.e., *Escherichia coli, Bifidobacterium longum, Bacteroides vulgatus, Clostridium ramosum, Peptostreptococcus productus* and *Lactobacillus gasseri.* These bacteria had originally been isolated from normal human feces, and represented the predominant strain of each group. Each bacterium was cultured for 72 h at 37°C in GAM semifluid medium (Nissui Co., Tokyo, Japan) and then homogenized with a stirrer without access of air. A 1-mL portion of the culture medium containing 10^8–10^9 bacteria/mL was administered to the rats. Conventional rats of the same strain and the same age were also examined.

The rats were fed a commercial diet (Oriental CMF Diet; Oriental Kobo Co., Tokyo, Japan) which had been sterilized by radiation with ^{60}Co (50 KGy). Feces from each rat were collected before inoculation and also each day after inoculation for 6 d. A portion of the feces collected on the last day was utilized to confirm that the

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Abbreviations: FT, Fourier transform; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; INADEQUATE, incredible natural abundance double-quantum transfer experiment; NMR, nuclear magnetic resonance.

TABLE 1

Fecal Bacterial Count in Monoassociated Rats Six Days After Inoculation

	Inoculated bacterial count log N/mL	Bacterial count log N/g wet weight feces
Escherichia coli	9.1	9.8
Bifidobacterium longum	8.4	9.7
Bacteroides vulgatus	8.4	10.6
Clostridium ramosum	8.6	9.4
Peptostreptococcus productus	8.5	9.7
Lactobacillus gasseri	8.5	9.4

bacterial counts were more than 10^9/g wet weight of feces (Table 1) using the method of Benno *et al.* (14), and the remainder was used for bile acid analysis.

Soon after the final collection of feces, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the bile duct was cannulated with a PE-10 polyethylene tube to collect bile for 30 min, with the rectal temperature being kept at 37°C by means of an electrical heating plate (15). After removing blood from the abdominal aorta to sacrifice the animals, the small intestine and the large intestine, including the cecum, were removed along with their contents.

Bile acid determination. Bile acids were extracted from bile with 20 vol of ethanol at 85–90°C for 10 min. After filtration, a portion of the extract was dried under a stream of nitrogen and subjected to PHP-LH-20 column chromatography (16) to obtain the fractions of free, glycine-conjugated, taurine-conjugated and sulfated bile acids.

The small and large intestinal contents were homogenized with 20 mL of distilled water, and a portion of the homogenates was lyophilized. Bile acids were extracted from the dry preparation with absolute ethanol at 85–90°C for 1 h, and the mixture was filtered. This extraction procedure was repeated three times (17), and the extracts were combined and dried. A solution in 90% ethanol was subjected to PHP-LH-20 column chromatography (16).

The feces were lyophilized and ground in a small mill. Bile acids were extracted from a portion of the feces (usually 0.5 g) by treatment with 15 mL of absolute ethanol at 85–90°C for 1 h, and a filtered solution was obtained (17). The extraction procedures were the same as those used for the intestinal contents.

The fractions obtained after PHP-LH-20 column chromatography were purified on a Sep-Pak C_{18} cartridge (Water Associates, Milford, MA) and then concentrated under reduced pressure (18). The sulfate fraction was subjected to solvolysis (19). The glycine- and taurine-conjugated bile acids were hydrolyzed using cholylglycine hydrolase (EC 3.5.1.24; Sigma, St. Louis, MO) (20), and the deconjugated bile acids were extracted with diethyl ether after acidification with 2 N hydrochloric acid.

The bile acids were methylated with freshly prepared diazomethane and then trifluoroacetylated with trifluoroacetic anhydride (17,21). The bile acid derivatives were quantified by gas–liquid chromatography utilizing a Hewlett Packard (Palo Alto, CA) gas-chromatograph, Model HP5890A, equipped with a hydrogen flame-ionization detector and an HP-7673A autoinjector. A capillary column (15 m × 0.25 mm i.d.) coated with DB-17 (J&W Scientific, Folsom, CA) was used. The column was programmed from 200–280°C at a rate of 5°C/min; the injection port was kept at 280°C, and the detector at 300°C.

RESULTS

Enzymatic hydrolysis of the compound eluted as peak A (compound A) with cholylglycine hydrolase and methylation with diazomethane produced a methyl ester. The ^{1}H NMR spectrum of the methyl ester derived from compound A was quite similar to that of the methyl ester of β-muricholic acid except for two additional signals at δ_H 5.978 (*dd*, J = 15.5 and 0.6 Hz, 23-H) and δ_H 7.080 (*dd*, J = 15.5 and 9.0 Hz, 22-H), as is compared in Table 2. The chemical shifts and couplings (J = 15.5 Hz) of these signals indicate the presence of an *E*-double bond with one hydrogen at the vicinal position. The down-field shifts of the 20-methyl signal (δ_H 1.076, *d*, J = 6.6 Hz, 21-H) and the ester methyl signal (δ_H 3.731, *s*, $COOCH_3$) suggest that the methyl ester of compound A is the methyl ester of β-muricholic acid having an *E*-double bond at C-22 and C-23. The presence of an α, β-unsaturated carboxy ester system is consistent with the observed UV absorption at 214 nm (22). The ^{1}H NMR spectrum of compound A is essentially the same as that of the methyl ester of compound A except for the presence of three signals at δ_H 3.531 (*t*, J = 6.7 Hz, CH_2SO_3H), δ_H 4.332 (*brq*, J = 6.0 Hz, CH_2N), and δ_H 8.705 (*t*, J = 5.8 Hz, NH) and the absence of the ester methyl signal. These three additional signals of compound A can be assigned to the hydrogens of the taurine moiety. Thus, compound A is proposed to be tauro-Δ^{22}-β-muricholic acid.

The ^{13}C NMR spectrum of β-muricholic acid obtained in [$^2H_5$]pyridine has been reported by Kuroki *et al.* (23). We have mostly confirmed the assignments of these signals by 2D INADEQUATE ^{13}C NMR spectroscopy (12,13,24) as shown in Figure 1. In the 2D INADEQUATE spectrum of β-muricholic acid, all connectivities of the carbons were established except for C-1, C-2, C-3 and C-4, whose signal intensity was too low. However, our assignments were not in agreement with those of Kuroki *et al.* (23) with respect to C-14 and C-17.

The ^{13}C NMR signals of the methyl ester of β-muricholic acid were assigned by chemical shift comparisons with β-muricholic acid. The chemical shift changes for C-1 through C-21 of the methyl ester vs. the acid are within 0.24 ppm. The changes for C-22 through C-24 are –0.70, –0.38 and –2.22 ppm, respectively, which are greater due to the methyl esterification (see Ref. 25, and references therein). The methyl ester methoxy signal appears at δ_C 51.25.

The ^{13}C NMR spectrum of the methyl ester of compound A resembles that of the methyl ester of β-muricholic acid except for the signals originating from the side-chain carbons. The ^{13}C NMR signals were assigned based on data reported for a number of bile acids (23;

TABLE 2

^{13}C and ^{1}H Nuclear Magnetic Resonance Data[a] of Bile Acid Derivatives in [$^{2}H_5$]Pyridine

	β-Muricholic acid	Methyl ester of β-muricholic acid	Methyl ester of compound A	Compound A
C-1	36.51	36.50	36.51	36.50
C-2	30.93	31.07	31.08	31.05
C-3	70.83	70.74	70.74	70.75
C-4	36.70	36.87	36.88	36.84
C-5	48.67	48.82	48.85	48.80
C-6	76.44	76.47	76.47	76.46
C-7	73.34	73.37	73.37	73.35
C-8	39.09	39.19	39.20	39.14
C-9	40.37	40.25	40.22	40.24
C-10	34.42	34.44	34.49	34.47
C-11	21.44	21.33	21.27	21.27
C-12	40.56	40.41	40.22	40.24
C-13	43.92	43.80	44.06	43.95
C-14	56.42[b]	56.30	56.06	56.11
C-15	27.80	27.79	27.84	27.82
C-16	29.05	28.89	28.91	29.10
C-17	55.60[b]	55.38	54.56	54.85
C-18	12.52	12.54	12.58	12.56
C-19	26.31	26.26	26.26	26.25
C-20	35.83	35.60	39.89	39.69
C-21	18.91	18.67	19.57	19.95
C-22	31.91	31.21[c]	155.40	149.50
C-23	31.91	31.53[c]	119.14	122.82
C-24	176.64	174.42	167.31	166.59
CH_2S		—	—	51.65
CH_2N		—	—	36.74
OCH_3		51.25	51.24	—
18-H		0.684 *s*	0.692 *s*	0.672 *s*
21-H		0.953 *d*	1.076 *d*	1.051 *d*
		(6.0 Hz)	(6.6 Hz)	(6.6 Hz)
19-H		1.432 *s*	1.450 *s*	1.421 *s*
OCH_3		3.662 *s*	3.731 *s*	—
7-H		3.780 *dd*	3.780 *dd*	3.763 *dd*
		(10.0 and 2.6 Hz)	(10.5 and 3.5 Hz)	(10.0 and 3.7 Hz)
3-H		3.884 *m*	—	3.880 *m*
6-H		4.053 *dd*	4.064 *dd*	4.053 *dd*
		(10.0 and 2.6 Hz)	(4.0 and 2.0 Hz)	(3.2 and 2.2 Hz)
23-H		—	5.978 *dd*	6.086 *d*
			(15.5 and 0.6 Hz)	(15.3 Hz)
22-H		—	7.080 *dd*	7.047 *dd*
			(15.5 and 9.0 Hz)	(15.3 and 8.8 Hz)
CH_2S		—	—	3.531 *t*
CH_2N				(6.7 Hz)
		—	—	4.332
NH	*brq*			
		—	—	(6.0 Hz)
				8.705 *t*
				(5.8 Hz)

[a]δ_C, δ_H, multiplicities and *J* (in parentheses). [b]The assignments reported in Reference 23 were revised based on the incredible natural abundance double-quantum transfer experiment shown in Figure 1. [c]These assignments may be reversed.

and Ref. 26 and references therein) and sterols (11, and for example, Ref. 27) and by taking into consideration chemical shift rules (25,28, and references therein) and known solvent effects in [^{2}H]chloroform and [$^{2}H_5$]pyridine (e.g., 28). The chemical shift changes for C-1 through C-16, and for C-18 and C-19, as well as for the methoxy carbon, between the methyl ester of compound A and the methyl ester of β-muricholic acid are within

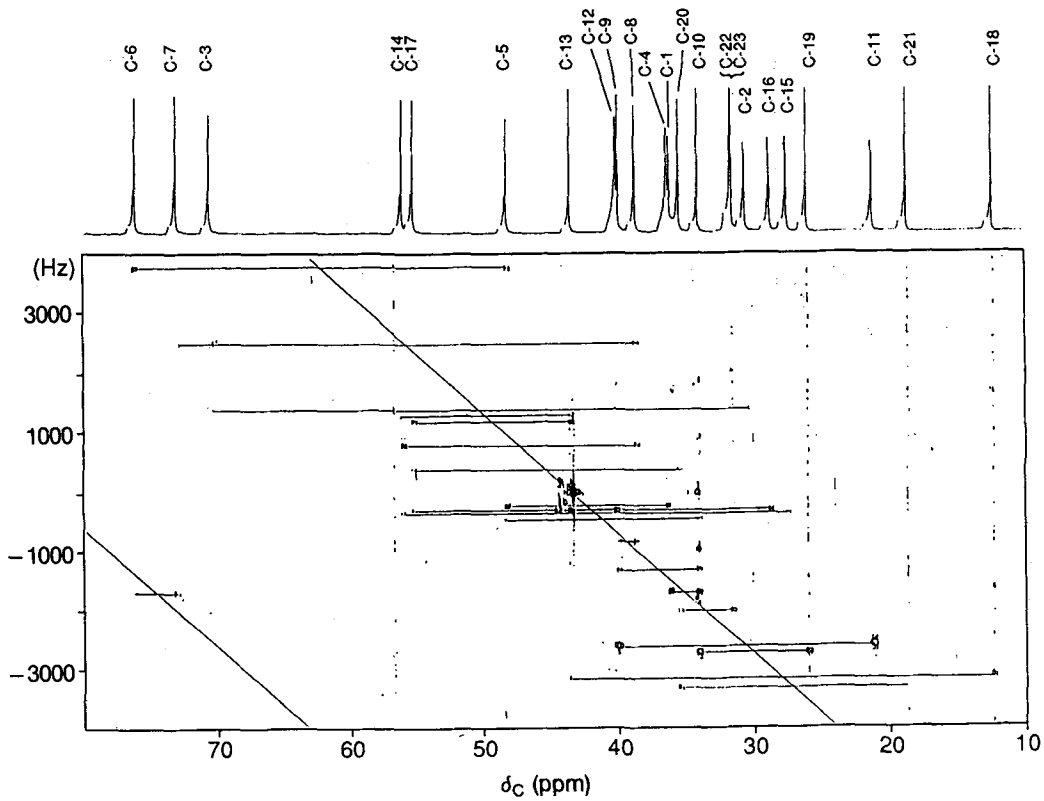

FIG. 1. 2D incredible natural abundance double-quantum transfer experiment spectrum of β-muricholic acid in [$^2H_5$]pyridine (for details, see text).

0.26 ppm. The differences for C-17 and for C-20 through C-24 are −0.82, +4.29, +0.90, +124.19, +87.61 and −7.11 ppm, consistent with the presence of a double bond (11,25,27). The *E* double bond in the methyl ester of compound A is also indicated by the ^1H-^1H spin-spin coupling of 15.5 Hz between 22-H and 23-H in the ^1H spectrum. Based on the data presented here, the methyl ester of compound A is confirmed to be methyl (22-*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oate.

A comparison of the ^{13}C NMR signals of compound A with those of its methyl ester derivative shows that instead of the ester methyl signal (δ_C 51.24), compound A has two signals at δ_C 36.74 and δ_C 51.65, which are assigned to the two carbons of the taurine moiety. The chemical shift changes for C-22, C-23 and C-24 between the methyl ester of compound A and compound A are −6.90, +3.62 and −0.72 ppm, respectively, which are consistent with a change from an α,β-unsaturated ester to an α,β-unsaturated amide. This confirms that compound A is tauro-(22-*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oate.

The data in Table 3 give the bile acid components in the feces of germ-free, monoassociated and conventional rats. The fecal bile acids in the germ-free rats were taurine-conjugated, while those in the conventional rats were almost completely deconjugated and only a trace amount of taurine conjugates was detected. *E. coli* did not detectably deconjugate bile acids; however, the other five bacteria caused deconjugation although their activity varied from 8 to 47% deconjugation. The total amounts of fecal bile acids in the germ-free and monoassociated rats were about half those in the conventional rats.

In conventional rats, several secondary bile acids were found in the feces, including deoxycholic, 7-oxo-deoxycholic, 12-oxo-lithocholic, lithocholic, hyodeoxycholic and ω-muricholic acids, in addition to the primary bile acids. In germ-free rats, the fecal bile acids consisted of only the primary bile acids; i.e., mainly cholic and β-muricholic acids and small amounts of chenodeoxycholic and α-muricholic acids. In the monoassociated rats, the pattern of fecal bile acids was almost the same as that in the germ-free rats, except that Δ^{22}-β-muricholic acid was detected in the feces of the rats inoculated with *B. vulgatus, C. ramosum, P. productus* or *L. gasseri* (Table 4).

The bile acid compositions in the large intestine, as shown in Table 5, were very similar to those in the feces. The bile acid compositions of the bile and the small intestine, as shown in Tables 6 and 7, were also very similar to each other. The ratio of cholic acid was high and that of β-muricholic acid was low in the bile and in the small intestine.

The percentages of the deconjugated form of cholic acid, β-muricholic acid and Δ^{22}-β-muricholic acid, in the small intestine, in the large intestine, and in the feces were calculated and are shown in Table 8. No deconjugated bile acid was detected in the bile of all the groups. Deconjugation of bile acid did not occur in the germ-free rats and in the rats inoculated with *E. coli* as reported

TABLE 3

Fecal Bile Acids in Germ-Free, Monoassociated and Conventional Rats[a]

	Before inoculation	Six days after inoculation					
	Total	Total	Unconjugated	Glycine	Taurine	Sulfated	Deconjugation ratio
	(mg/d/rat)	(mg/d/rat)					
Germ-free	3.9 ± 0.2	3.6 ± 0.1	nd[b]	nd	3.6 ± 0.1	nd	0.0 ± 0.0
E. coli	3.4 ± 0.1	2.1 ± 0.1	nd	nd	2.1 ± 0.1	nd	0.0 ± 0.0
B. longum	3.9 ± 0.3	3.5 ± 0.2	0.8 ± 0.1	nd	2.6 ± 0.4	nd	24.1 ± 1.8
B. Vulgatus	3.4 ± 0.4	3.4 ± 0.2	1.3 ± 0.1	nd	2.2 ± 0.1	nd	39.6 ± 0.6
C. ramosum	3.2 ± 0.1	2.7 ± 0.4	1.3 ± 0.2	nd	1.4 ± 0.2	nd	46.8 ± 1.1
P. productus	3.3 ± 0.1	3.1 ± 0.2	1.1 ± 0.1	nd	2.0 ± 0.2	nd	34.7 ± 2.3
L. gasseri	3.4 ± 0.2	3.2 ± 0.3	0.3 ± 0.0	nd	3.0 ± 0.2	nd	7.7 ± 0.4
Conventional	—	6.4 ± 0.7	6.4 ± 0.7	nd	trace[c]	nd	99.6 ± 0.2

[a]The data are the means ± SEM of five rats; total, total bile acid; unconjugated, unconjugated bile acid; glycine, glycine conjugated bile acid; taurine, taurine conjugated bile acid; sulfated, sulfated bile acid. Deconjugation ratio: (unconjugated bile acid)/(unconjugated bile acid + conjugated bile acid) × 100. See Table 1 for complete spellings of bacteria names. [b]nd, Not detectable. [c]Trace amounts of less than 0.1 mg/d/rat.

TABLE 4

Fecal Bile Acid Composition in Germ-Free, Monoassociated and Conventional Rats[a]

(%)	Germ-free	*E. coli*	*B. longum*	*B. vulgatus*	*C. ramosum*	*P. productus*	*L. gasseri*	Conventional
3α12α[b]	nd[c]	nd	nd	nd	nd	nd	nd	34.4 ± 4.4
3α7α12α[b]	47.0 ± 0.7	48.6 ± 1.1	52.4 ± 0.5	46.2 ± 0.9	49.5 ± 1.0	52.9 ± 0.2	45.3 ± 0.9	2.3 ± 1.4
3α12α7=O[b]	nd	nd	nd	nd	nd	nd	nd	trace[d]
3α7α12=O[b]	nd	nd	nd	nd	nd	nd	nd	nd
3α12=O[b]	nd	nd	nd	nd	nd	nd	nd	11.3 ± 1.5
3α[e]	nd	nd	nd	nd	nd	nd	nd	2.3 ± 0.3
3α6β7α[e]	1.1 ± 0.1	1.3 ± 0.1	0.5 ± 0.1	2.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	1.9 ± 0.0	1.6 ± 0.1
3α7α[e]	1.5 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	3.4 ± 0.6	0.7 ± 0.1	0.8 ± 0.2	1.2 ± 0.1	trace
3α6α[e]	nd	nd	nd	nd	nd	nd	nd	1.3 ± 0.8
3α6β7β[e]	50.2 ± 0.7	47.2 ± 0.7	45.5 ± 0.5	32.7 ± 1.7	41.6 ± 0.4	41.4 ± 0.9	48.9 ± 1.0	16.7 ± 3.1
3α6α7β[e]	nd	nd	nd	nd	nd	nd	nd	12.2 ± 2.1
3α7=O[e]	nd	nd	nd	nd	nd	nd	nd	nd
Δ^{22} · 3α6β7β[e]	nd	nd	nd	13.5 ± 0.7	6.2 ± 1.0	1.7 ± 0.3	1.3 ± 0.1	nd
Unknown	trace	1.2 ± 0.3	0.5 ± 0.0	1.8 ± 0.3	0.6 ± 0.2	1.8 ± 0.9	1.5 ± 0.1	16.8 ± 0.6

[a]The data are the means ± SEM of five rats. See Table 1 for complete spellings of bacteria names.[b]Cholic acid group. [c]nd, Not detectable. [d]Trace amounts of less than 0.5%. [e]Chenodeoxycholic acid group.

TABLE 5

Large Intestinal Bile Acid Composition in Germ-Free, Monoassociated and Conventional Rats[a]

(%)	Germ-free	*E. coli*	*B. longum*	*B. vulgatus*	*C. ramosum*	*P. productus*	*L. gasseri*	Conventional
3α12α[b]	nd[c]	nd	nd	nd	nd	nd	nd	32.2 ± 0.9
3α7α12α[b]	47.3 ± 0.6	50.3 ± 0.6	58.3 ± 1.9	38.8 ± 2.4	49.7 ± 0.7	53.6 ± 2.1	48.0 ± 1.6	4.6 ± 1.3
3α12α7=O[b]	nd	trace[d]	1.2 ± 0.5	trace	trace	trace	nd	nd
3α7α12=O[b]	nd	nd	trace	nd	trace	nd	nd	nd
3α12=O[b]	nd	nd	nd	nd	nd	nd	nd	trace
3α[e]	nd	nd	nd	nd	nd	nd	nd	1.2 ± 0.6
3α6β7α[e]	trace	trace	trace	trace	trace	trace	trace	trace
3α7α[e]	2.2 ± 0.1	2.1 ± 0.6	1.8 ± 0.1	2.7 ± 0.3	1.4 ± 0.2	1.5 ± 0.3	1.8 ± 0.5	1.6 ± 0.2
3α6α[e]	nd	nd	nd	nd	nd	nd	nd	nd
3α6β7β[e]	48.4 ± 0.3	46.2 ± 1.4	35.6 ± 1.8	35.8 ± 1.1	37.6 ± 0.7	40.4 ± 2.4	47.4 ± 1.5	32.5 ± 2.4
3α6α7β[e]	nd	nd	nd	nd	nd	nd	nd	18.3 ± 4.7
3α7=O[e]	nd	nd	nd	nd	nd	nd	nd	nd
Δ^{22} · 3α6β7β[e]	nd	nd	nd	13.9 ± 1.1	8.5 ± 0.2	2.1 ± 0.7	0.9 ± 0.4	nd
Unknown	1.9 ± 0.9	1.5 ± 1.0	2.3 ± 0.2	8.1 ± 0.8	1.7 ± 1.0	1.8 ± 0.1	1.5 ± 0.7	9.4 ± 1.6

[a]The data are the means ± SEM of five rats. See Table 1 for complete spellings of bacteria names. [b]Cholic acid group. [c]nd, Not detectable. [d]Trace amounts of less than 0.5%. [e]Chenodeoxycholic acid group.

TABLE 6

Biliary Bile Acid Composition in Germ-Free, Monoassociated and Conventional Rats[a]

(%)	Germ-free	*E. coli*	*B. longum*	*B. vulgatus*	*C. ramosum*	*P. productus*	*L. gasseri*	Conventional
3α12α[b]	nd[c]	nd	nd	nd	nd	nd	nd	3.3 ± 0.6
3α7α12α[b]	79.4 ± 0.7	69.1 ± 1.6	78.7 ± 0.7	75.0 ± 2.4	68.8 ± 0.8	72.9 ± 0.9	70.4 ± 1.6	79.5 ± 0.7
3α12α7=O[b]	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	1.6 ± 0.2
3α7α12=O[b]	nd	nd	nd	nd	nd	nd	nd	nd
3α12=O[b]	nd	nd	nd	nd	nd	nd	nd	nd
3α[d]	nd	nd	nd	nd	nd	nd	nd	nd
3α6β7α[d]	trace[e]	0.7 ± 0.1	trace	0.7 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
3α7α[d]	3.7 ± 0.1	2.7 ± 0.2	3.6 ± 0.1	3.6 ± 0.3	3.2 ± 0.1	2.5 ± 0.5	3.4 ± 0.2	3.9 ± 0.3
3α6α[d]	nd	nd	nd	nd	nd	nd	nd	nd
3α6β7β[d]	14.2 ± 0.5	24.3 ± 1.7	13.9 ± 0.6	16.6 ± 2.1	23.8 ± 0.6	21.1 ± 0.9	22.3 ± 1.9	7.7 ± 0.7
3α6α7β[d]	nd	nd	nd	nd	nd	nd	nd	nd
3α7=O[d]	nd	nd	nd	nd	nd	nd	nd	nd
Δ^{22} · 3α6β7β[d]	nd	nd	nd	0.8 ± 0.2	0.6 ± 0.1	trace	nd	0.7 ± 0.1
Unknown	2.2 ± 0.2	2.7 ± 0.1	3.1 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	2.3 ± 0.3	2.9 ± 0.1	2.8 ± 0.3

[a]The data are the means ± SEM of five rats. See Table 1 for complete spellings of bacteria names. [b]Cholic acid group. [c]nd, Not detectable. [d]Chenodeoxycholic acid group. [e]Trace amounts of less than 0.5%.

TABLE 7

Small Intestinal Bile Acid Composition in Germ-Free, Monoassociated and Conventional Rats[a]

(%)	Germ-free	*E. coli*	*B. longum*	*B. vulgatus*	*C. ramosum*	*P. productus*	*L. gasseri*	Conventional
3α12α[b]	nd[c]	nd	nd	nd	nd	nd	nd	2.3 ± 0.5
3α7α12α[b]	81.3 ± 0.8	81.7 ± 0.8	80.5 ± 1.0	82.2 ± 0.8	81.9 ± 0.8	83.4 ± 0.6	78.4 ± 0.7	78.3 ± 1.6
3α12α7=O[b]	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.0	1.4 ± 0.2
3α7α12=O[b]	nd	nd	nd	nd	nd	nd	nd	nd
3α12=O[b]	nd	nd	nd	nd	nd	nd	nd	nd
3α[d]	nd	nd	nd	nd	nd	nd	nd	nd
3α6β7α[d]	trace[e]	trace	trace	trace	trace	trace	trace	1.6 ± 0.2
3α7α[d]	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.1 ± 0.4	2.6 ± 0.1	2.6 ± 0.2
3α6α[d]	nd	nd	nd	nd	nd	nd	nd	nd
3α6β7β[d]	12.7 ± 0.6	12.2 ± 0.6	12.6 ± 0.5	10.9 ± 0.5	11.4 ± 0.6	10.8 ± 0.3	13.9 ± 0.4	10.5 ± 1.4
3α6α7β[d]	nd	nd	nd	nd	nd	nd	nd	trace
3α7=O[d]	nd	nd	nd	nd	nd	nd	nd	nd
Δ^{22} · 3α6β7β[d]	nd	nd	nd	0.5 ± 0.0	trace	nd	nd	1.0 ± 0.1
Unknown	2.4 ± 0.2	2.8 ± 0.2	3.1 ± 0.4	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.2	3.5 ± 0.2	2.8 ± 0.4

[a]The data are the means ± SEM of five rats. See Table 1 for complete spellings of bacteria names. [b]Cholic acid group. [c]nd, Not detectable. [d]Chenodeoxycholic acid group. [e]Trace amounts of less than 0.5%.

TABLE 8

Deconjugation of Bile Acids in Small Intestine, Large Intestine and Feces[a]

(%)	Germ-free	*E. coli*	*B. longum*	*B. vulgatus*	*C. ramosum*	*P. productus*	*L. gasseri*	Conventional
Small intestine								
3α7α12α[b]	nd[c]	nd	trace[d]	nd	nd	nd	trace	35.2 ± 13.3
3α6β7β[e]	nd	nd	nd	nd	nd	nd	nd	27.2 ± 13.1
Δ^{22} . 3α6β7β[e]	nd	nd	nd	nd	nd	nd	nd	23.4 ± 3.0
Large intestine								
3α7α12α[b]	nd	nd	95.2 ± 0.6	19.3 ± 0.8	67.9 ± 2.1	86.2 ± 1.2	48.2 ± 3.1	100.0 ± 0.0
3α6β7β[e]	nd	nd	12.9 ± 2.4	85.1 ± 1.7	55.0 ± 2.1	62.1 ± 1.5	27.5 ± 1.0	100.0 ± 0.0
Δ^{22} . 3α6β7β[e]	nd	nd	nd	55.8 ± 1.4	45.4 ± 2.1	8.4 ± 8.4	nd	nd
Feces								
3α7α12α[b]	nd	nd	42.2 ± 2.1	nd	52.4 ± 1.1	40.9 ± 2.5	14.7 ± 0.7	88.5 ± 11.5
3α6β7β[e]	nd	nd	3.4 ± 1.5	78.3 ± 0.7	43.8 ± 0.6	27.4 ± 2.1	4.5 ± 0.1	100.0 ± 0.0
Δ^{22} . 3α6β7β[e]	nd	nd	nd	66.3 ± 1.7	37.6 ± 0.7	nd	nd	nd

[a]The data are the means ± SEM of five rats. See Table 1 for complete spellings of bacteria names. [b]Cholic acid group. [c]nd, Not detectable. [d]Trace amounts of less than 0.5%. [e]Chenodeoxycholic acid group.

earlier (4). Hardly any deconjugated bile acid was found in the small intestine of the monoassociated rats. Most of the deconjugated bile acids were detected in the large intestine and in the feces in the monoassociated rats, except for the rats inoculated with *E. coli*. In the conventional rats, the bile acids were deconjugated (about 30%) even in the small intestine. *Bacteroides vulgatus*, preferentially deconjugated tauro-β-muricholic acid, and *B. longum* deconjugated taurocholic acid. *C. ramosum, P. productus* and *L. gasseri* deconjugated both bile acids.

Δ^{22}-β-Muricholic acid was not formed at all in the germ-free rats and in the monoassociated rats inoculated with *E. coli* and *B. longum*. The rats inoculated with *B. vulgatus, C. ramosum, P. productus* or *L. gasseri* formed Δ^{22}-β-muricholic acid, which was mainly found in the large intestine and in the feces. A small amount of tauro-Δ^{22}-β-muricholic acid was detected in the bile and in the small intestine of rats given a single inoculation of *B. vulgatus, C. ramosum* or *P. productus*. In conventional rats, Δ^{22}-β-muricholic acid was not detected in the large intestine or the feces, but accounted for about 1% of total bile acids in the bile and in the small intestine. Some of the bacterial strains examined in the present study, especially *B. longum, C. ramosum* and *P. productus*, deconjugated taurocholic acid but Δ^{22}-cholic acid could not be detected after treatment with these strains.

DISCUSSION

The present study has shown that Δ^{22}-β-muricholic acid was formed in monoassociated rats inoculated with *B. vulgatus, C. ramosum, P. productus* or *L. gasseri*. It was interesting that these strains of human intestinal bacteria could desaturate β-muricholic acid even though this bile acid is not present in human bile and feces. Its formation seems to be correlated with the deconjugation activity of tauro-β-muricholic acid in feces. *Bacteroides vulgatus* showed the highest activity in the deconjugation of tauro-β-muricholic acid and also showed the highest ratio of Δ^{22}-β-muricholic acid to total bile acid. The germ-free rats and rats monoassociated with *E. coli* did not deconjugate tauro-β-muricholic acid and formed no Δ^{22}-β-muricholic acid. The rats monoassociated with *B. longum* could deconjugate tauro-β-muricholic acid, but the activity was low, and Δ^{22}-β-muricholic acid could not be detected.

Since deconjugation does not occur in the small intestine, Δ^{22}-β-muricholic acid should be formed after deconjugation in the large intestine in the monoassociated rats. Δ^{22}-β-Muricholic acid in the bile and in the small intestine of the monoassociated rats was taurine-conjugated, suggesting that it was absorbed from the large intestine and excreted into the bile after conjugation with taurine in the liver.

In the conventional rats, a small amount of Δ^{22}-β-muricholic acid was found in the bile and in the small intestine. About 30% of tauro-β-muricholic acid had been deconjugated in the small intestine probably because of bacterial upward colonization. This may explain why Δ^{22}-β-muricholic acid was detected in the bile and in the small intestine. Δ^{22}-β-Muricholic acid was not detected in the large intestine or in the feces of conventional rats, even though β-muricholic acid had been completely deconjugated. It is difficult to explain this discrepancy; however, we can presume that the anaerobic conditions in the large intestine would be different between monoassociated and conventional rats. It is also possible that there are other bacteria in the large intestine of conventional rats that can reduce Δ^{22}-β-muricholic acid.

The presence of a monounsaturated β-muricholic acid in rat bile has been reported in several studies (9,10,29,30). Kuriyama *et al.* (31) detected β-muricholic acid with a double bond in the side chain in rat bile by gas chromatography/mass spectrometry (GC/MS). Although *B. vulgatus, C. ramosum, P. productus* and *L. gasseri* did deconjugate tauro-β-muricholic acid and formed Δ^{22}-β-muricholic acid, *B. longum, C. ramosum, P. productus* and *L. gasseri* did deconjugate taurocholic acid but formed no Δ^{22}-cholic acid in our experiments.

Murata *et al.* (32) noted that they could not detect cholic acid with a double bond in the side chain in rat bile by GC/MS analysis. Robben *et al.* (10) also mentioned that they could not find Δ^{22}-cholic acid in rats inoculated with *Clostridium* sp. Cl_8. The results suggest that the mechanism of taurocholic acid deconjugation is different from that of tauro-β-muricholic acid deconjugation. The mechanism by which the double bond is introduced is not known, although desaturation between C-22 and C-23 may be achieved in a manner similar to that which occurs in the β-oxidation of fatty acid via acyl-CoA dehydrogenase.

Deconjugation, 7-dehydroxylation, oxidation–reduction and epimerization activities have all been reported for intestinal bacteria (1–3), but deconjugation activity is most widely found in many bacteria, including *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Peptostreptococcus, Lactobacillus* and *Streptococcus*. In the present study the deconjugation activity for rat endogenous bile acids was detected in *B. longum, B. vulgatus, C. ramosum, P. productus* and *L. gasseri* but not in *E. coli*. *Bacteroides vulgatus* preferentially deconjugated tauro-β-muricholic acid, and *B. longum* preferentially deconjugated taurocholic acid, while *C. ramosum, P. productus* and *L. gasseri* deconjugated both.

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Sponge Fatty Acids. 3. Occurrence of Series of n-7 Monoenoic and *iso*-5,9 Dienoic Long-Chain Fatty Acids in the Phospholipids of the Marine Sponge *Cinachyrella* aff. *schulzei* Keller[1]

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The fatty acid composition of phospholipids from the New Caledonian sponge *Cinachyrella* aff. *schulzei* Keller was studied. More than 60 fatty acids were identified as methyl esters and *N*-acyl pyrrolidides by gas chromatography and gas chromatography/mass spectrometry. Two isoprenoid fatty acids also were shown to be present, namely 4,8,12-trimethyltridecanoic and 5,9,13-trimethyltetradecanoic acids. The unusual 6-tetradecenoic, 6-pentadecenoic, 12-nonadecenoic and 26-methylheptacosanoic (*iso*-28:0) acids were found for the first time in sponge phospholipids. A series of six n-7 monoenoic long-chain fatty acids (C_{23} to C_{28}) were identified, including the rare 16-tricosenoic, 18-pentacosenoic and 21-octacosenoic acids. Fifteen fatty acids possessing the typical 5,9 dienoic moiety accounted for 30% of the total fatty acid mixture. Two new fatty acids were identified, namely 5(Z)-octacosenoic and 27-methyl-5(Z),9(Z)-octacosadienoic (*iso*-5,9-29:2). Based on gas chromatography/Fourier transform infrared experiments, the double bonds were assigned the (Z) configuration.
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Marine sponges are rich sources of long-chain phospholipid fatty acids possessing unique branched or substituted carbon chains probably arising from unique biosynthetic pathways (1–4). Many aspects of sponge phospholipid chemistry and biochemistry have been reviewed by Djerassi and Lam (5). We recently described the phospholipid fatty acid composition of *Cinachyrella alloclada* from the Senegalese coast (6), and have continued our investigations on sponge species belonging to the *Cinachyrella* genus collected in different geographical locations to provide comparative data that also may serve as chemotaxonomic criteria. Some isoprenoid fatty acids have been reported to occur in sponges, especially 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD), which rarely occurs in abundance; however, it amounted to about 20% of total fatty acids in *C. alloclada* and *Spheciospongia vesparium* (6,7). It was in the phospholipids of *C. alloclada* that two different isoprenoid fatty acids were first found, namely the 4,8,12-TMTD (6) and the unusual 5,9,13-trimethyltetradecanoic acid (5,9,13-TMTD). *Iso*- and *anteiso*-saturated long-chain fatty acids are not common in nature, but some have been reported to be associated with sponge phospholipids (8). Straight-chain monoenoic fatty acids with Δ5, Δ6 or Δ8 unsaturation are also not very common in the phospholipids of sponges. Thus, only a few examples of Δ5 monoenoic long-chain fatty acids (LCFA) have been reported (9,10) even though they are known to be intermediates in the biosynthesis of 5,9-dienoic demospongic acids (11,12).

Our search of novel fatty acids in sponge phospholipids is also intended to find potential biosynthetic intermediates of sponge fatty acid metabolism. The biosynthetic route operative in marine sponges involves an elongation process in the n-7 monoenoic series from short-chain homologues, which extends only to C_{26} unsaturated fatty acids (12,13). The Senegalese sponge *Higginsia tethyoides* contains a complete series of such n-7 monoenoic LCFA, but all are α-methoxy-substituted analogues (2). 16-Tricosenoic acid was found first in *C. alloclada* (6). This acid and several other monoenoic LCFA have been found by Carballeira *et al.* (14) in *Amphimedon compressa* and *Mycale laevis*. In this respect, our findings of naturally occurring 23-triacontenoic and 9,23-triacontadienoic acids in *Trikentrion loeve* would seem to be of considerable interest (1), as these acids have been postulated to be intermediates in the biosynthesis of the relatively abundant 5,9,23-triacontatrienoic acid (15). One feature common to many sponge fatty acids is the occurrence of the 5,9-dienoic system in fatty acids, with even and odd long carbon chains. However, very few sponges studied to date have been shown to contain many of these acids (16,17). Brominated demospongic fatty acids are rare and, to our knowledge, only three have been described to date (18,19).

We report here the phospholipid fatty acid composition of the sponge *Cinachyrella* aff. *schulzei* obtained from the lagoon of Noumea, New Caledonia.

EXPERIMENTAL PROCEDURES

Specimens of *Cinachyrella* aff. *schulzei* Keller (1891) (Demospongia, Tetractinomorpha, Spirophorida, Tetillidae) were collected by hand in Canal Woodin, Noumea, New Caledonia, at a depth of 25–30 m in late 1990. The sponge, referenced as MNHN R 1467 New Caledonia, is

[1]For part 2 of this series, see Reference 1.

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Abbreviations: ECL, equivalent chain length; FAME, fatty acid methyl ester(s); GC/MS, gas chromatography/mass spectrometry; GC/FT–IR, gas chromatography/Fourier transform infrared; LCFA, long-chain fatty acid(s); MS, mass spectrum; TLC, thin-layer chromatography; 4,8,12-TMTD, 4,8,12-trimethyltridecanoic acid; 5,9,13-TMTD, 5,9,13-trimethyltetradecanoic acid.

80 × 70 × 60 mm with porocalices 6 × 3 to 13 × 11 mm; its skeleton has an hispidation, 5 mm; sigmaspires, 22 μm; oxeas, 4.3 mm; small oxeas, 200–260 μm; anatriaenes, 5 mm, with studded or knobby clades, 25 μm, or with normal clades, 35–50 μm; protriaenes like anatriaenes; several prodiaenes. A similar species is *C. hirsuta* Dendy (1889).

The sponges were washed in sea water, carefully cleaned, cut into small pieces and lyophilized. Sponge pieces were ground in a Waring blender, using chloroform/methanol (1:1, vol/vol), and steeped twice in this solvent for 24 h (room temperature). The combined extracts yielded the crude total lipids. Phospholipids were separated from other lipids by column chromatography on silica gel (70–230 mesh) using hexane, chloroform, acetone and methanol (phospholipids) as successive eluents. About 55 mg of phospholipids was recovered from 90 g of sponge (dry weight). Phospholipid composition was analyzed by thin-layer chromatography (TLC) using chloroform/methanol/water (65:25:4, by vol) as eluent, and fractions were identified by comparison with phospholipid standards. Phospholipid fatty acids were converted to methyl esters by reaction (30 min under reflux) with methanolic hydrogen chloride (20), and the residue was dissolved in hexane and purified by column chromatography (silica gel; hexane/diethyl ether, 10:1, vol/vol). The resulting methyl esters were analyzed by gas–liquid chromatography using a Carlo Erba 4130 chromatograph (Milano, Italy) and a nonpolar OV-1 silica capillary column (A.M.L.-Chromato, Limoges, France) (25 m × 0.32 mm i.d., 0.40 μm film thickness); hydrogen was used as carrier gas (0.5 bar; split ratio, 5:100). Standard fatty acid methyl esters (from C_{20} to C_{31}) and standard phospholipid samples were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acyl pyrrolidide derivatives were prepared by treatment of methyl esters with pyrrolidine/acetic acid (10:1, vol/vol) under reflux (2 h) and were purified by TLC on 0.5-mm layers of silica gel with hexane/diethyl ether (1:2, vol/vol) as developing solvent. Fatty acid methyl esters were hydrogenated by stirring (4 h) at ambient pressure and temperature dissolved in methanol in the presence of catalytic amounts of platinum (IV) oxide (Adam's catalyst). Combined gas chromatography/mass spectrometry (GC/MS) was performed on a Hewlett-Packard HP-5890 instrument linked to an HP 9000/345 integrator (Palo Alto, CA). The GC column was a 0.32 mm × 30 m fused silica capillary column coated with DB-1 (0.25 μm film thickness). The carrier gas was helium. Column temperature was programmed from 180 to 310°C, at 3°C/min, for methyl esters and pyrrolidides.

Gas chromatography/Fourier transform infrared (GC/FT-IR) spectra were measured in Marseille, France, on a 20SxB Nicolet spectrometer (Madison, WI), interfaced with a 5300 Mega Carlo Erba chromatograph (flame-ionization detector additional detection) fitted with an on-column injector. The GC/IR light pipe is a gold-coated borosilicate cylinder (1 mm × 16 cm) with KBr windows. A medium range Hg-Cd-Te liquid-nitrogen cooled detector was used. The GC capillary column was a 60 m × 0.32 mm i.d. fused silica (J & W Scientific, Folsom, CA) coated with DB-1 phase (0.25 μm film thickness). The flow rate of the helium carrier gas (140 kPa) was about 2 mL/min. The column was then directed to the GC/IR light pipe through an inlet stainless steel glass lined transfer line maintained at 300°C. The oven was initially set at 70°C with a 2 min isotherm and then programmed to reach 130°C at 15°C/min, followed by a rate of 15°C/min to reach 290°C, and then maintained at this temperature for 40 min. For the experiment, the light pipe was heated to 293°C. Vapor phase IR spectra were recorded with a resolution of 8 cm^{-1} over the range of 4,000–650 cm^{-1}; 16 data scans were collected, and co-added per data file (2.03 for each spectrum). The IR reconstructed chromatogram was done using the Gram-Schmidt algorithm.

RESULTS

The major phospholipid classes were shown to be phosphatidylethanolamine and phosphatidylserine as judged by TLC analysis. Many of the fatty acids were identified by comparing their equivalent chain length (ECL) values as fatty acid methyl esters (FAME) with those of known compounds and with fatty acids from mixtures of known composition, as well as by co-injection with commercial standards (normal LCFA up to 31:0). Capillary GC analysis of a hydrogenated aliquot also provided information useful for identification (*iso* and *anteiso* compounds) of unsaturated fatty acids. The complete list of phospholipid fatty acids from *C.* aff. *schulzei* is given in Table 1.

LCFA with more than 22 carbons accounted for more than 50% of all acids. The major LCFA were 25-methyl-5,9-hexacosadienoic (*iso*-27:2; 7.2%), 19-hexacosenoic (26:1; 6.8%), 5,9,21-octacosatrienoic (28:3; 5.8%) and 26-methyl-5,9-heptacosadienoic (*iso*-28:2; 3.6%) acids. Unsaturated LCFA comprised about 90% of the total LCFA. Fifteen LCFA containing the 5,9 diene system accounted for 30% of total; this is the greatest number of Δ5,9 dienoic acids found to date in a marine sponge. However, for many acids, GC/MS data on pyrrolidide derivatives were obtained to confirm the structures and to determine the double bond and branching positions (21). The mass spectral data of some of the most interesting fatty acids are as follows:

8-Heptadecenoic acid pyrrolidide. MS *m/z* (rel. intensity), 321 (M^+, 2.0), 292 (1.3), 278 (1.8), 266 (1.5), 264 (2.0), 251 (2.2), 250 (4.4), 249 (3.1), 237 (1.3), 236 (2.9), 223 (1.8), 222 (1.6), 208 (1.5), 196 (0.7), 195 (1.8), 194 (2.2), 182 (4.1), 168 (4.3), 167 (2.0), 155 (2.6), 154 (2.8), 140 (5.7), 126 (39.6), 113 (85.4), 98 (46.5), 70 (45.8), 55 (100).

12-Nonadecenoic acid pyrrolidide. MS *m/z* (rel. intensity), 349 (M^+, 2.0), 334 (0.3), 320 (0.9), 306 (1.6), 292 (1.6), 278 (2.2), 264 (1.8), 252 (0.8), 250 (1.2), 239 (0.9), 238 (1.1), 224 (1.2), 222 (0.9), 210 (1.3), 196 (2.5), 182 (3.1), 168 (3.5), 154 (1.5), 141 (1.4), 140 (4.1), 126 (37.1), 113 (100), 98 (17.6), 70 (37.2), 55 (74.5).

18-Pentacosenoic acid pyrrolidide. MS *m/z* (rel. intensity), 433 (M^+, 3.1), 418 (0.3), 404 (0.8), 390 (1.7), 376 (1.6), 363 (3.5), 362 (3.1), 348 (2.2), 334 (1.2), 322 (1.3),

TABLE 1

Major Phospholipid Fatty Acids from *Cinachyrella* aff. *schulzei*[a]

Fatty acid	Symbol	ECL (FAME)	Abundance (%)
Dodecanoic	12:0	12.00	0.7
Tridecanoic	13:0	13.00	0.5
6-Tetradecenoic[b]	14:1	13.70	0.3
Tetradecanoic	14:0	14.00	2.6
4,8,12-Trimethyltridecanoic	16:0	14.49	8.8
14-Methyltetradecanoic	15:0	14.63	0.5
6-Pentadecenoic[b]	15:1	14.72	0.2
Pentadecanoic	15:0	15.00	0.8
5,9,13-Trimethyltetradecanoic	17:0	15.39	0.5
6-Hexadecenoic	16:1	15.74	0.7
9-Hexadecenoic	16:1	15.79	2.4
Hexadecanoic	16:0	16.00	10.3
10-Methylhexadecanoic	17:0	16.42	0.5
8-Heptadecenoic	17:1	16.82	0.6
Heptadecanoic	17:0	17.00	0.6
9,12-Octadecadienoic	18:2	17.52	0.6
16-Methylheptadecanoic	*i*-18:0	17.66	1.0
9-Octadecenoic	18:1	17.75	2.1
11-Octadecenoic	18:1	17.78	1.6
Octadecanoic	18:0	18.00	6.9
12-Nonadecenoic[b]	19:1	18.84	1.2
Heneicosanoic	21:0	21.00	0.5
Docosanoic	22:0	22.00	0.6
16-Tricosenoic	23.1	22.79	0.3
Tricosanoic	23:0	23.00	0.5
21-Methyltricosanoic	*a*-24:0	23.72	1.1
17-Tetracosenoic	24:1	23.79	2.2
Tetracosanoic	24:0	24.00	0.8
23-Methyl-5,9-tetracosadienoic	*i*-25:2	24.12	0.4
18-Pentacosenoic	25:1	24.81	1.0
24-Methyl-5,9-pentacosadienoic	*i*-26:2	25.08	0.8
5,9-Hexacosadienoic	26:2	25.43	2.6
17-Hexacosenoic	26:1	25.68	2.2
19-Hexacosenoic	26:1	25.79	6.8
Hexacosanoic	26:0	26.00	0.8
25-Methyl-5,9-hexacosadienoic	*i*-27:2	26.06	7.2
24-Methyl-5,9-hexacosadienoic	*a*-27:2	26.17	1.5
5,9-Heptacosadienoic	27:2	26.40	2.3
20-Heptacosenoic	27:1	26.80	0.5
Heptacosanoic	27:0	27.00	0.5
26-Methyl-5,9-heptacosadienoic	*i*-28:2	27.05	3.6
5,9,21-Octacosatrienoic	28:3	27.15	5.8
5,9,23-Octacosatrienoic	28:3	27.25	0.7
5,9-Octacosadienoic	28:2	27.40	2.6
26-Methylheptacosanoic[b]	*i*-28:0	27.63	1.2
5-Octacosenoic[c]	28:1	27.75	0.6
21-Octacosenoic	28:1	27.83	0.8
27-Methyl-5,9-octacosadienoic[c]	*i*-29:2	28.08	0.5
Bromo-5,9-heptacosadienoic	Br-5,9-27:2	28.18	0.5
5,9-Nonacosadienoic	29:2	28.38	1.1
5,9,23-Tricontatrienoic	30:3	29.10	0.9
5,9,x-Tricontatrienoic	30:3	29.20	trace
Tricontanoic	30:0	30.00	0.6

[a]Several common minor (<0.5%) fatty acids have also been identified, including *i*-14:0, *i*-15:0, *a*-15:0, *i*-16:0, *i*-17:0, *a*-17:0, *i*-19:0, 19:0, *a*-20:0, 20:0, *a*-23:0, *br*-24:0, 25:0, *a*-27:0, 28:0. *i, iso*; *a, anteiso*; *br*, branched; Br, bromine. ECL, equivalent chain length; FAME, fatty acid methyl esters.

[b]Not previously found in sponges.

[c]Not previously found in nature.

308 (1.1), 294 (1.5), 280 (2.3), 266 (1.6), 252 (1.7), 238 (1.9), 224 (1.7), 210 (1.1), 208 (0.9), 196 (0.8), 182 (3.0), 168 (3.9), 155 (1.6), 154 (1.8), 140 (5.3), 126 (56.5), 113 (100) 98 (38.5), 70 (100).

5-Octacosenoic acid pyrrolidide. MS *m/z* (rel. intensity), 476 (MH^+, 1.6), 446 (0.4), 432 (0.3), 431 (0.4), 418 (1.8), 404 (0.4), 390 (0.3), 376 (3.2), 362 (0.4), 348 (0.8), 334 (0.5), 322 (0.5), 320 (0.3), 308 (0.6), 292 (0.7), 278 (1.8), 266 (1.7), 264 (1.4), 252 (1.3), 250 (1.2), 236 (1.7), 222 (1.6), 210 (1.8), 209 (2.9), 208 (2.1), 195 (3.8), 194 (4.5), 180 (2.0), 168 (1.1), 166 (1.4), 152 (7.3), 140 (4.7), 126 (20.9), 113 (67.3), 98 (30.4), 70 (78.4), 55 (100).

21-Octacosenoic acid pyrrolidide. MS *m/z* (rel. intensity), 475 (M^+ 3,4), 446 (0.3), 432 (0.4), 430 (0.5), 418 (1.2), 404 (1.2), 390 (0.6), 376 (0.8), 364 (0.3), 350 (0.5), 340 (0.3), 336 (0.5), 322 (0.6), 308 (1.4), 295 (0.7), 294 (1.0), 280 (2.7), 266 (1.3), 252 (0.7), 238 (0.6), 224 (0.7), 210 (1.3), 198 (1.4), 196 (1.6), 182 (2.2), 180 (2.0), 168 (1.8), 154 (1.6), 140 (3.5), 126 (19.8), 113 (100), 98 (17.5), 70 (30.2), 55 (59.3).

27-Methyl-5,9-octacosadienoic acid pyrrolidide. MS *m/z* (rel. intensity), 487 (M^+, 2.3), 472 (0.8), 444 (1.7), 435 (0.8), 417 (0.8), 375 (1.7), 360 (2.2), 346 (1.8), 318 (1.6), 300 (0.7), 276 (2.5), 262 (2.4), 248 (1.2), 235 (2.2), 234 (1.2), 221 (2.0), 207 (4.6), 192 (2.0), 180 (20.5), 168 (2.5), 166 (2.4), 154 (2.0), 152 (2.4), 140 (5.2), 126 (17.9), 113 (100), 98 (21.8), 85 (12.3), 71 (14.5).

Bromo-5,9-heptacosadienoic acid pyrrolidide. MS *m/z* (rel. intensity), 458 ($[M - Br]^+$, 5.5), 260 (4.2), 258 (4.0), 210 (1.2), 180 (6.3), 126 (8.3), 113 (100), 98 (18.4), 85 (11.6), 72 (23.0), 70 (23.0).

Four homologous series of fatty acids were recognized (Fig. 1) when the retention times of the LCFA (as methyl esters) were plotted vs. the carbon numbers. These were the straight-chain acids, n-7 monoenoic acids, normal Δ5,9 dienoic acids and *iso*-5,9-dienoic acids.

Saturated fatty acids. We readily identified two saturated isoprenoid acids, 4,8,12-TMTD (8.8%) and 5,9,13-TMTD acids (0.5%). GC mobilities and GC/MS data for the methyl esters and pyrrolidides of these compounds were described in a recent paper (6). 26-Methylheptacosanoic acid (*iso*-28:0) was also present (1.2%) and readily identified as its methyl ester. It had an ECL of 27.63, and the molecular ion peak was at *m/z* 438, with additional fragments at *m/z* 74 (base peak), 87 and 395

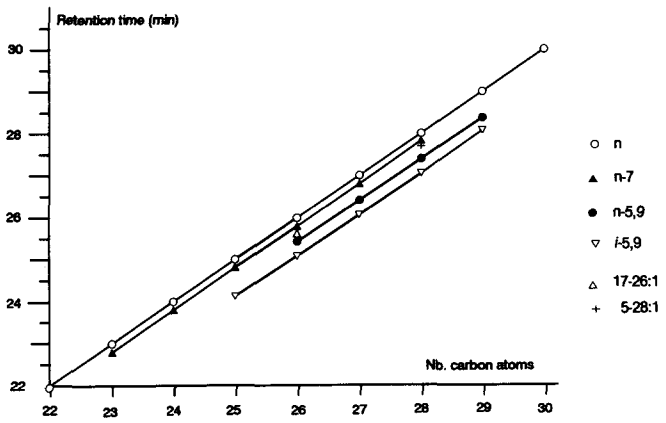

FIG. 1. Plot of retention time (min) vs. number of carbon atoms for four series of fatty acid methyl esters from *Cinachyrella* aff. *schulzei*. The monoenoic acids 5-28:1 and 17-26:1 are located outside the n-7 monoenoic straight line.

(M – 43). The GC peak did not change upon catalytic hydrogenation.

Monoenoic fatty acids. Three interesting monoenoic fatty acids were identified in *C.* aff. *schulzei* (14:1, 15:1, 16:1). Although they were present in small amounts, these acids could readily be identified based on GC mobility and GC/MS data. On the other hand, they were converted to 14:0, 15:0 and 16:0 derivatives, respectively, upon catalytic hydrogenation. Mass spectra of the pyrrolidide derivatives showed the key fragments at m/z 154 and 166 (with a difference of 12 amu occurring between the C-5 and C-6 fragments), clearly indicating Δ6 unsaturation, and molecular ions at m/z 279 (14:1), 293 (15:1) and 307 (16:1), respectively. No diminished homologous fragment was observed, thus excluding branching. Another rare fatty acid identified, namely 8-heptadecenoic acid (0.6%), had an ECL value as methyl ester (FAME) of 16.82 and a molecular ion peak at m/z 282. The mass spectrum of the corresponding pyrrolidide derivative showed a 12 amu difference between peaks m/z 182 and 194 (Δ8 unsaturation). The unusual 12-nonadecenoic acid (1.2%) was readily identified as its methyl ester had an ECL value of 18.84 and a molecular ion peak at m/z 310. Upon catalytic hydrogenation, this compound was converted to the nonadecanoic acid methyl ester. The mass spectrum of the pyrrolidide derivative exhibited a molecular ion peak at m/z 349 and key fragments at m/z 238 and 250 (Δ12 unsaturation). An interesting monoenoic LCFA was found in *C.* aff. *schulzei.* Its methyl ester had an ECL value of 27.75 and a molecular peak at m/z 436, indicating that it was an octacosenoic acid methyl ester. The MS of the pyrrolidide exhibited a molecular ion peak at m/z 476 (MH^+) and key fragments at m/z 140 and 152, indicating desaturation at Δ5 (Fig. 2). This 5-octacosenoic acid (5-28:1, 0.6%) had not been found previously in any natural source.

In addition to the usual 17-tetracosenoic and 19-hexacosenoic acids of the n-7 monoenoic series, we identified the 16-tricosenoic acid that we recently found in *C. alloclada* (6), as well as three other very rare fatty acids lately found for the first time in Caribbean sponges (14) and characterized by GC/MS of the dimethyl disulfide adducts, namely 18-pentacosenoic, 20-heptacosenoic and 21-octacosenoic.

The first acid (18-25:1, 1.0%) showed and ECL value as FAME of 24.81 and a molecular ion peak at m/z 394. It was converted to pentacosanoic acid methyl ester upon catalytic hydrogenation. The mass spectrum of its pyrrolidide showed a molecular peak at m/z 433 and key fragments at m/z 322 and 334, indicating Δ18 unsaturation. We were thus able to identify the 18-pentacosenoic acid previously described to occur in *M. laevis* (14). The second acid, 21–28:1, (0.8%) was identified in the same way; its methyl ester had an ECL value of 27.83 and a molecular peak at m/z 436 and, converted to octacosanoic acid methyl ester upon catalytic hydrogenation, was characterized by comparison with an authentic sample. The MS of its pyrrolidide derivative showed a molecular ion peak at m/z 475 and key fragments at m/z 364 and 376, indicating Δ21 unsaturation. This fatty acid was the 21-octacosenoic acid already identified in *A. compressa* (14). The third fatty acid was identified as a heptacosenoic acid (27:1, 0.5%); its methyl ester had an ECL value of 26.80 and a molecular ion peak at m/z 422. The corresponding peak disappeared upon catalytic hydrogenation. Unfortunately, a suitable MS of the pyrrolidide derivative was not obtained owing to the small amount available and the fact that the spectrum was obscured by fragments derived from the stationary silicone phase. When retention time vs. carbon number was plotted, this compound fell exactly in line with the other n-7 monoenoic FAME (Fig. 1), especially the homologous 19–26:1 and 21–28:1. In Figure 1 the second line includes the heptacosenoic acid in question, but not closely related monoenoic acids, such as 17-hexacosenoic and 5-octacosenoic (as FAME). Thus, the 20-heptacosenoic structure was only tentatively assigned to this fatty acid, which was previously identified in *A. compressa* (14). The FT-IR spectra of the n-7 monoenoic methyl ester (Fig. 3) showed absorptions at 3013 cm^{-1} (stretching frequency of the ethylenic bond) and 703 cm^{-1} (out-of-plane bending vibration) character-

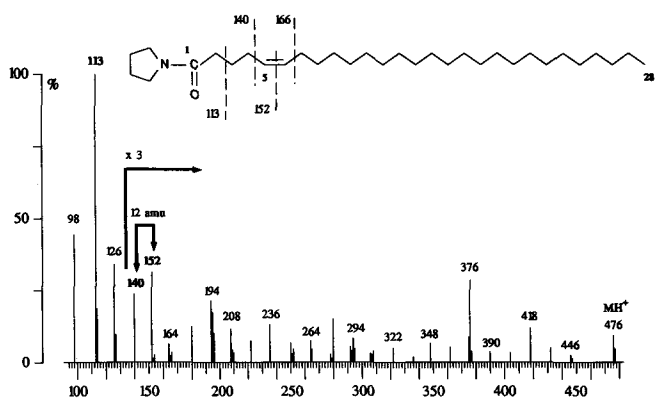

FIG. 2. Mass spectrum of 5-octacosenoic acid pyrrolidide.

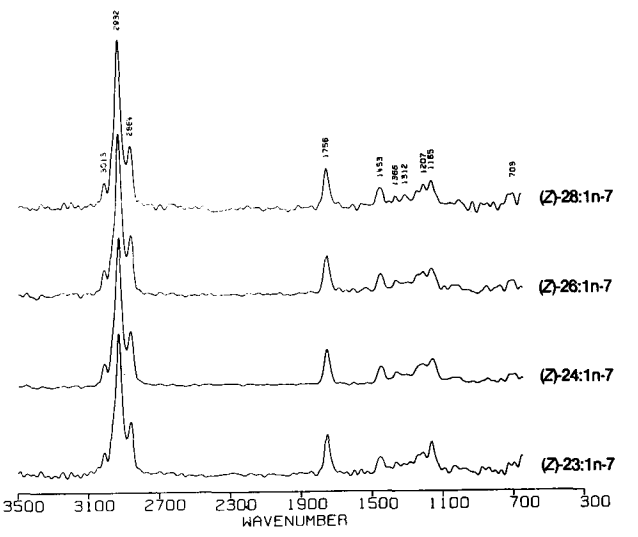

FIG. 3. Gas chromatography/Fourier transform infrared spectra of several n-7 monoenoic fatty acid methyl esters.

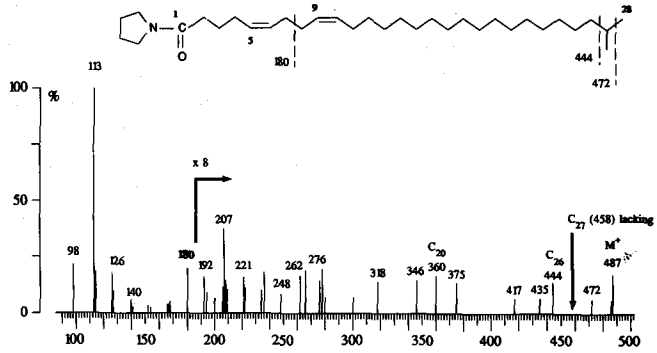

FIG. 4. Mass spectrum of 27-methyl-5,9-octacosadienoic acid pyrrolidide showing the typical fragmentation peak at *m/z* 180 and lack of a peak at *m/z* 458 (C_{27} fragment).

istic of a *cis* configuration (no absorption near 960 cm^{-1}) (22,23).

Long-chain Δ5,9 dienoic fatty acids. One main feature of *C.* aff. *schulzei* is the presence of fifteen long-chain fatty acids possessing typical sponge Δ5,9 unsaturation. This pattern was readily identified (MS), as all these fatty acids afforded the characteristic peaks at *m/z* 81 and 180 as methyl esters and pyrrolidides, respectively. Catalytic hydrogenation yielded the corresponding saturated FAMEs that had a normal *iso* or *anteiso* hydrocarbon chain for the two latter fractional chain lengths of 0.62–0.65 and 0.72–0.75, respectively. Most of them have been already found in sponges. In addition to the rare 23-methyl-5,9-tetracosadienoic acid (24,25), we also identified another compound belonging to the series of *iso*-5,9-dienoic fatty acids for which the MS of the FAME (ECL 28.08) exhibited a molecular ion peak at *m/z* 448, indicating a 29:2 structure. The base peak was the typical peak at *m/z* 81, indicating a 5,9-dienoic structure. This FAME gave the 27-methyloctacosanoic acid methyl ester (*iso*-29:0, ECL 28.64) upon catalytic hydrogenation. The MS of the corresponding pyrrolidide derivative (Fig. 4) showed a molecular ion peak at *m/z* 487 (29:2) and the typical major ion at *m/z* 180 (5,9-29:2). Methyl branching at the C-27 position was indicated by significant peaks at *m/z* 444 (C_{26}) and *m/z* 472 (C_{28}) and by absence of the C_{27} fragment at *m/z* 458. Thus a novel fatty acid was identified, namely 27-methyl-5,9-octacosadienoic (*iso*-5,9-29:2).

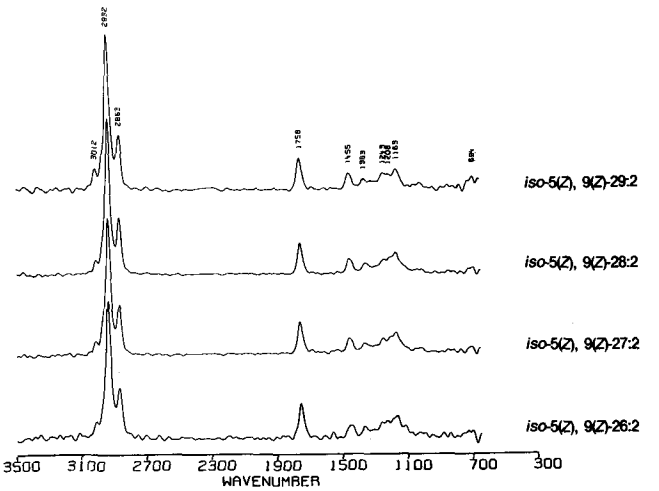

FIG. 5. Gas chromatography/Fourier transform infrared spectra of several *iso*-5,9 long-chain fatty acid methyl esters.

A complete series of *iso*-5,9 long-chain fatty acids (C_{25}–C_{29}) occurs in *C.* aff. *schulzei.* In Figure 5, the double bonds are in *cis* configuration as all infrared spectra exhibited absorptions at 3012 cm^{-1} and 694 cm^{-1} (out-of-plane bending vibration) (22,23). No absorption band was present near 960 cm^{-1}. Finally, it is noteworthy that this novel FAME fell in line with the *iso*-5,9-dienoic methyl esters in Figure 1. Moreover, an interesting 5,9-dienoic fatty acid with an ECL value of 28.18 was also detected. The MS of the methyl ester showed a peak at *m/z* 74 (McLafferty rearrangement) and a major peak at *m/z* 81 characteristic of the 5,9-dienoic moiety. In addition, the MS showed a series of peaks at *m/z* 331, 345, 355, 373 and 404, which closely corresponded to those obtained with the 5,9-heptacosadienoic acid methyl ester (*m/z* 332, 346, 356, 374 and 406). These data suggest the presence of a labile substituent that can be easily lost under electron impact, according to the observations of Lam *et al.* (19). The MS of the pyrrolidide derivative was very simple, with the usual base peak at *m/z* 113 and the typical peak at *m/z* 180, confirming the presence of a 5,9-dienoic pattern. However, the usual series of homologous fragments was absent. There was a significant peak at *m/z* 458 and a doublet of equal intensity at *m/z* 258 and 260, suggesting that the labile substituent was, in fact, a bromine atom. Thus, the double allylic fragmentation between C-7 and C-8 (typical of 5,9-dienoic acid pyrrolidides) gave the usual ion at *m/z* 180 after bromine loss and peaks at *m/z* 258 and 260 with the bromine substituent intact, so that the peak at *m/z* 458 corresponded to the molecular ion peak after bromine loss. These data imply that the point of bromine attachment was between C-2 and C-7. The bromine substituent is at a vinylic position in all known brominated fatty acids (18,19,26), especially in brominated 5,9-dienoic long-chain fatty acids (18,19). Unfortunately, we were unable to identify the precise position of bromine attachment by nuclear magnetic resonance experiments, due to the small amount of sample available. The acid could be identical to the LCFA (5*E*,9*Z*)-6-bromo-5,9-heptacosadienoic recently described in *Petrosia* sp. (27).

Δ5,9 Trienoic LCFA. We identified three Δ5,9-trienoic acids (MS of FAME and *N*-acyl pyrrolidides) already known to occur in sponges, particularly the major one (5.8%), 5,9,21-octacosatrienoic acid, in addition to the common 5,9,23-tricontatrienoic acid. Traces of another tricontatrienoic acid were also detected. This compound was readily identified as methyl ester possessing the typical Δ5,9 unsaturation pattern (ECL 29.20, M^+ at *m/z* 460 and prominent peak at *m/z* 81), but the exact position of the third double bond could not be assigned due to the very small amount of pyrrolidide available.

DISCUSSION

The co-occurrence of two saturated isoprenoid fatty acids in a marine sponge was an interesting finding. It had been previously suggested (7) that it would be unlikely that the two isoprenoid fatty acids would occur in the same sponge. We initially found two such acids, namely 4,8,12-TMTD and 5,9,13-TMTD acids, in the Senegalese sponge *C. alloclada* (6), and two other isoprenoid fatty acids, 4,8,12-trimethyl-13:0 and phytanic acid, have recently been identified in *Dysidea fragilis* (25). It is surprising that *C.* aff. *schulzei*, from a quite different location, also contains these two isoprenoid fatty acids. The former acid is frequently found, though not in all sponges, whereas the latter is a very rare compound identified here only for the second time in a sponge. In addition, all specimens studied of the Senegalese sponge *C. kükenthali*, either from deep or shallow waters, contained both these isoprenoid acids (28). The co-occurrence of these two isoprenoid fatty acids would thus seem to characterize a sponge belonging to the genus *Cinachyrella*. Work is in progress in our laboratory to verify this feature for other *Cinachyrella* species collected from different geographical locations and to elucidate the biosynthetic pathways. The very rare 26-methylheptacosanoic acid (*iso*-28:0) was identified here for the first time in a sponge. It had already been mentioned as a hydrogenation product of *iso*-5,9-28:2 acid found in some sponges (16,17). Only a few natural sources of *iso*-28:0 were previously known, and none was associated with natural phospholipids, although it was recently reported to occur as a minor component of the total lipid fatty acids from sulfate-reducing bacteria (29).

Two unusual monoenoic fatty acid patterns were found in *C.* aff. *schulzei*. The identification of the rare 8-heptadecenoic acid is the second report of this acid in a sponge after its recent identification by Carballeira and Restituyo in *A. complanata* (30). We reported the rare 8-hexadecanoic acid in *C. alloclada* (6). These acids could have a dietary or a symbiotic origin. We also identified small amounts of 6-tetradecenoic, 6-pentadecenoic and 6-hexadecenoic acids. The latter acid had earlier been found in the sponge *A. complanata* (30), but we have not been aware of a report of 6-tetradecenoic and 6-pentadecenoic acids in any marine sponge. The 6-monoenoic acids were recently found to be biomarkers of planktonic input (31). 6-Heptadecenoic and 6-nonadecenoic acids were identified in the marine sponges *Strongylophora durissima* (10) and *Calyx niceaensis* (32), respectively, and 14-methyl-6-pentadecenoic acid was detected in *Tethya aurantia* (33). Several marine organisms, such as turtles (34), sunfish (35), whales (36), sea anemones (37), jellyfish and gorgonians (38), have been shown to contain *trans*- or *cis*-6-hexadecanoic acid. These 6-monoenoic fatty acids may also be of bacterial origin. As previously suggested by Carballeira and Maldonado for *Eurypongia rosea* (39), we think that a Δ6 desaturase may be operative in *C.* aff. *schulzei*.

One of the main findings of the present study is the occurrence in *C.* aff. *schulzei* of a series of n-7 LCFA possessing even- and odd-numbered carbon chains. The odd monoenoic series contains the 12-nonadecenoic, 13-tricosenoic, 18-pentacosenoic and the tentatively assigned 20-heptacosenoic acids. The rare 12-nonadecenoic acid, previously identified from *Thiobacillus* strains (40), is reported here for the first time as being present in sponge phospholipids. In fact, few nonadecenoic acids are known to occur in nature, the most common examples being 9-19:1 and 11-19:1. The 6-nonadecenoic acid was found in sponges (27), and we reported the 13-nonadecenoic acid, probably of bacterial origin, in *C. alloclada* (6). 16-Tricosenoic acid was previously identified in the Senegalese sponge *C. alloclada* (6), together with 10-heptadecenoic acid and an uncharacterized pentacosenoic acid. The 18-pentacosenoic acid described here was already found in *M. laevis* (14). Thus, from *Cinachyrella* sponges, we isolated a complete series of odd n-7 monenoic fatty acids (17–27 carbon atoms) with the exception of 14-21:1, which would also be expected to be present in sponge phospholipids. A complete series of n-7 monoenoic LCFA is known to occur in human brain lipids (41). The phospholipids of the Senegalese sponge *Higginsia tethyoides* (2) contain acids 2-methoxy-18-pentacosenoic, 2-methoxy-20-heptadecenoic and 2-methoxy-21-octacosenoic. This seems to suggest that in *H. tethyoides* methoxyl is introduced in a last step. In addition to the known 17-24:1 and 19-26:1 acids, *C.* aff. *schulzei* was shown to contain the 21-28:1 acid, which was encountered before only in *A. compressa*. The acid was recently synthesized (42). An interesting octacosenoic isomer was also characterized, namely 5-octacosenoic acid, not previously found in a sponge. The co-occurrence of 21-octacosenoic, 5-octacosenoic and 5,9,21-octacosatrienoic acids suggests a role for the first of these acids as a biosynthetic intermediate toward the 5,9,21-octacosatrienoic acid *via* elongation from palmitoleic or vaccenic acid. The 21-28:1 acid was postulated as such an intermediate (43), but in *C.* aff. *schulzei* Δ9 desaturation appears to be the last biosynthetic step. In addition to the well known 5,9,23-30:3, we detected another distinct 30:3 possessing typical Δ5,9 desaturation. As we found both 5,9,21-28:3 and 5,9,23-28:3 acids in *C.* aff. *schulzei*, it is likely, and in accordance with our GC data, that this tricontatrienoic fatty acid is the 5,9,25-30:3 that we recently identified in *T. loeve* (1). Work is in progress in our laboratory on other *Cinachyrella* sponge specimens to identify this new tricontatrienoic acid. Only one of the tricontatrienoic acids has been reported to date in marine sponges, the relatively abundant 5,9,23-30:3 acid. The finding of a brominated demospongic acid also seems of interest. Lam *et al.* (19) recently demonstrated that biological bromination occurs at the final step in the biosynthesis of such fatty acids.

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METHOD

A Simple and Precise Method for the Routine Determination of Platelet-Activating Factor in Blood and Urine

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A simple and precise method is described for the measurement of platelet-activating factor (PAF) in blood and urine. The method involves the isolation of PAF from blood samples by two successive steps. In the first step, blood proteins are precipitated with ethanol and the "free" PAF, i.e., the PAF which is extractable with ethanol, is recovered. In the second step, "bound" PAF, i.e., PAF not extractable with ethanol, is extracted from the protein precipitate with chloroform/methanol/water. The extraction of PAF from urine samples requires only the ethanol extraction step. "Free" and "bound" PAF are then each fractionated by silicic acid column chromatography, and the methanol/water eluent containing PAF is then further fractionated by high-performance liquid chromatography using an isocratic solvent system of acetonitrile/methanol/water. PAF is then quantitated by measuring its ability to induce platelet aggregation in an aggregometer. Application of the method to blood and urine samples from twenty-three healthy volunteers revealed PAF levels in blood of 140–480 pg/mL (630–254.4 pg "free" PAF/mL and 64–225.6 pg "bound" PAF/mL), and of 1.2–4.0 pg PAF/mL in urine. The method overcomes various technical problems and was shown to be very precise. It should prove useful for monitoring PAF levels in various disease conditions.
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Platelet-activating factor (PAF), i.e., 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1–3), is one of the most potent mediators of acute allergic and inflammatory reactions (4). PAF is synthesized and released from a variety of eucaryotic (4,5) and procaryotic (6,7) cells and has been detected in human body fluids (8–11).

To investigate the possible role of PAF in the pathogenesis of various immune complex diseases and systemic allergic reactions, much attention has been paid to the estimation of PAF levels in biological fluids. Differences between PAF levels in normal biological fluids and in samples gathered in several disease states are consistent with the hypothesis that PAF is involved in the pathogenesis of a number of diseases. PAF levels in biological fluids have been investigated in cases of allergic rhinitis (8), allergic reactions induced by drugs (9), post-transfusion reactions (10), gastrointestinal cancer (11), lupus erythematosus (12), allergic inflammation in experimental animals (13), anephric individuals (14), peritoneal inflammation (15) and cirrhosis of the liver (16).

In this type of study, the purification of PAF from other contaminants in the samples examined remains a major difficulty. Incomplete purification of PAF affects PAF analyses and can unpredictably alter the PAF values obtained by up to one or two orders of magnitude. Current methods for the purification of native PAF are based on the extraction with organic solvents and isolation by thin-layer chromatography systems (8,13,14). These procedures are time-consuming, and their success largely depends on the type of tissue studied. Ethanol extraction was found to be advantageous for the isolation of PAF and its precursors from human polymorphonuclear neutrophils, monocytes and lymphocytes (17) as well as from plasma and whole blood (18). In blood, a significant portion of native PAF is known to be bound to lipoproteins. Deproteinization with HCl and a solid-phase purification step have previously been used in the assay of PAF in human polymorphonuclear leukocytes stimulated with ionophor A23187 (19). High-performance liquid chromatography (HPLC) is then commonly used as the final step in the purification of PAF(8–11,17–19).

In an effort to develop a routine method for the assay of PAF, that would be generally applicable, we found that ethanol extraction followed by chloroform/methanol/water extraction when combined with a suitable system of silicic acid column chromatography and semi-preparative HPLC gave precise and highly reproducible results. The present method is simple enough to permit the analysis of large numbers of samples and the separate estimation of native "bound" PAF and "free" PAF. The method may facilitate clarifying the role of PAF in various disorders.

MATERIALS AND METHODS

Samples. Blood and urine samples (5 mL) were collected from twenty-three healthy volunteers. The samples were immediately poured into absolute ethanol and refrigerated.

Materials and reagents. All reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). HPLC solvents were purchased from Rathburn (Walkerburn, Peebleshire, United Kingdom). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA). Semisynthetic PAF and lysoPAF (80% C_{16} PAF and 20% C_{18} PAF) were synthesized in our laboratory as previously described (1). [^{3}H]PAF (NET 910) was

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Abbreviations: BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TLC, thin-layer chromatography.

purchased from NEN (Dupont, Boston, MA). Silicic acid, 35–70 mesh, ASTM 7733 (Merck) was used for column chromatography.

Silicic acid column preparation. The silicic acid was washed with water and methanol and activated overnight at 120°C. The glass column (10 mm i.d.), was slurry-packed (20 cm height) using methanol/water (1:1.5, vol/vol). Lipid samples were applied in 1–2 mL of the same solvents.

Instrumentation. HPLC was done on a Perkin-Elmer Series 3B liquid chromatograph (Norwalk, CT) equipped with a Rheodyne (Berkley, CA) 7105 loop valve injector (175 µL). A Model 551 UV-VIS spectrophotometer (Perkin-Elmer) with a special 8-µL flow microcell was used as detector. The spectrophotometer was connected to a Model 2 integrator (Perkin-Elmer) and a Model 550 recorder (Perkin-Elmer). The cation exchange column used was a SS 10 µm Partisil, 25 cm ¥ 4.6 mm i.d., PXS 10/25 SCX from Whatman (Clifton, NJ). PAF-induced platelet aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer linked to an Omniscribe recorder (Houston, TX). Radioactivity was measured in a 1209 RackBeta-Flexivial β-counter (LKB-Pharmacia, Turku, Finland).

Biological assays. The PAF assay was done by measuring the aggregatory activity toward washed rabbit platelets (1) and by comparing the PAF-induced aggregation and/or [^{3}H]serotonin secretion with that induced by known concentrations of semisynthetic PAF (1).

[^{3}H]PAF estimation. In separate experiments, [^{3}H]PAF was added to several blood samples (5 mL), to test and calibrate the method. Radioactivity was then measured in the eluents from (i) the ethanol extraction, (ii) the silicic acid column and (iii) the HPLC column. In addition, [^{3}H]PAF was also added to some blood samples (5 mL) before the entire procedure was carried out, and remaining radioactivity was measured at the end of the procedure. Samples were dried under a nitrogen stream before radioactivity was measured in the β-counter.

RESULTS

Extraction. Blood samples were poured immediately after collection into 20 mL of absolute ethanol in 50-mL centrifuge glass tubes. After thorough mixing, the tubes were centrifuged at 1,000 ¥ *g* for 20 min. The supernatant containing "free" PAF was collected by decantation. The remaining pellet was mixed with 25 mL of chloroform/methanol/water (1:2:0.8, by vol) and, after thorough swirling, the tubes were centrifuged as above. The supernatant that was collected by decantation contained the "bound" PAF. Appropriate volumes of chloroform and water were then added to both supernatants to arrive at a final chloroform/methanol/water ratio of 1:1:0.9 (by vol) (20). Both chloroform phases were collected separately and evaporated to dryness at 35°C in a flash evaporator. The dry residues were transferred into test tubes using small volumes of chloroform/methanol (1:1, by vol), and the samples were dried under a stream of nitrogen. Urine samples were poured immediately after collection into 20 mL of absolute ethanol in 50-mL glass centrifuge tubes. After thorough mixing, the tubes were centrifuged at 1,000 × *g* for 20 min. The precipitate was discarded, and the supernatant was portioned with chloroform (20). The chloroform phase was dried as described for blood samples.

Silicic acid column chromatography. Each of the extracts was redissolved in 1–2 mL of methanol and applied to a silicic acid column with a 300 mL solvent dispenser. The column was eluted with 45 mL of methanol/water (1:1.5, vol/vol) followed by 50 mL of methanol/water (2:1, vol/vol). The initial 45-mL eluate containing the bulk of protein and other nonlipid components was discarded. The following 50-mL eluates, which contain PAF, were collected and portioned by adding chloroform and methanol to arrive at a final ratio of chloroform/methanol/water of 1:1:0.9 (by vol). The chloroform phase containing PAF and lipid impurities was evaporated to dryness at 35°C in a flash evaporator. The residue was transferred into a test tube using a small volume of chloroform/methanol (1:1, vol/vol) and dried under a stream of nitrogen.

Semi-preparative HPLC. The dry residue obtained in the previous step was redissolved in 50 µL of chloroform/methanol (1:1, vol/vol) and subjected to HPLC *via* the loop injector. The tube was washed with 50 µL of solvent, and the entire sample was injected into the column. PAF and other lipids were isocratically eluted with acetonitrile/methanol/water (300:150:35, by vol) at a flow rate of 1.5 mL per min; detection was at 206 nm. PAF was eluted at 12 min (21). The retention time of PAF is initially determined by starting each series of analyses with the injection of a standard sample containing sphingomyelin (SM) and lysophosphatidylcholine. Using [^{3}H]PAF, it was shown that in our system elution of PAF started at about 60 s after elution (Fig. 1A) and that essentially all radioactivity (98–100%) was recovered within 1.5 mL of eluate. The eluates collected before and after the PAF sample (1.5 mL each) did not cause inhibition of platelet aggregation induced by PAF under standard conditions. Hence, collection of the PAF fraction can begin about 30 s after elution of SM and can be completed after collecting 2–3 mL of eluate to avoid any losses.

Biological assay. The PAF fraction obtained by HPLC was dried down and then redissolved in 100 µL of a solution containing 2.5 mg bovine serum albumin (BSA) per mL saline. For the biological assay, appropriate volumes of the BSA solution were added to the aggregometer cuvettes as described in the Materials and Methods section.

Application of the method to blood and urine samples. Blood samples from ten healthy volunteers and urine samples from five healthy volunteers were used to determine PAF according to the present method. As shown in Table 1, native PAF levels measured ranged from 140–480 pg/mL of blood (63–254.4 pg "free" PAF/mL and 64–225.6 pg "bound" PAF/mL). PAF levels in urine samples ranged from 1.2–4.0 pg/mL of urine as summarized in Table 2.

[^{3}H]PAF recovery. [^{3}H]PAF was added to some blood samples and used internal standard. Recovery of

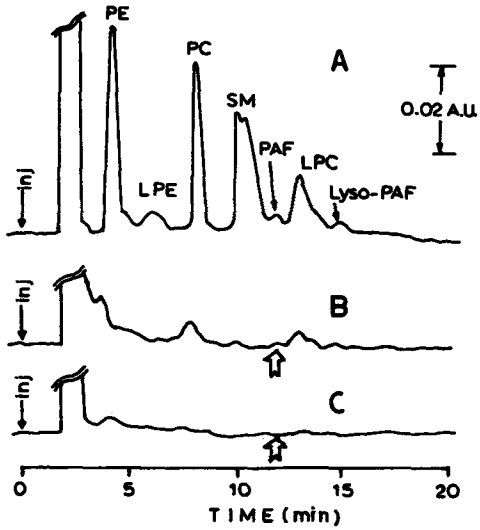

FIG. 1. High-performance liquid chromatography separation of platelet-activating factor (PAF) in biological samples and standards. A, standard lipids, PAF and lyso-PAF. B, a representative chromatogram from a blood sample as obtained either after ethanol extraction of whole blood or chloroform/methanol/water extraction of the ethanolic precipitate and purification by silicic acid column chromatography. C, a representative chromatogram of a urine sample as obtained after ethanol extraction and silicic acid column chromatography. Chromatographic conditions are as described in the text. The open arrows indicate the retention time of PAF as determined by biological assay. Abbreviations: Inj, injection; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine; a.u., absorbance unit.

radioactivity was 95% in ethanol extracts, 98% after silicic acid column chromatography and 98% after HPLC. The recovery at the end of the entire purification procedure was 96%.

TABLE 1

Native PAF Assay on Blood Samples

	Silicic acid chromatography + HPLC			Silicic acid chromatography
Subject number	"Free" PAF (pg/mL)	"Bound" PAF (pg/mL)	"Total" PAF (pg/mL)	"Total" PAF (pg/mL)
1	117.0	143.0	260.0	6.8
2	220.0	220.0	440.0	8.8
3	163.2	176.8	340.0	2.2
4	254.4	225.6	480.0	—
5	80.0	120.0	200.0	—
6	96.0	64.0	160.0	—
7	63.0	77.0	140.0	—
8	140.3	205.84	346.14	—
9	157.5	220.5	378.0	—
10	138.2	192.4	330.6	—
11	—[a]	—	—	13.8
12	—	—	—	13.6
13	—	—	—	9.8
14	—	—	—	6.8

[a]Platelet-activating factor (PAF) assays have not been carried out. HPLC, high-performance liquid chromatography.

TABLE 2

Native PAF Assay on Urine Samples

	Silicic acid chromatography + HPLC			Silicic chromatography
Subject number	"Free" PAF (pg/mL)	"Bound" PAF (pg/mL)	"Total" PAF (pg/mL)	"Total" PAF (pg/mL)
1	2.6	0	2.6	—
2	2.0	0	2.0	—
3	4.0	0	4.0	—
4	1.2	0	1.2	—
5	3.0	0	3.0	—
6	—[a]	—	—	4.0
7	—	—	—	13.6
8	—	—	—	13.8
9	—	—	—	10.4

[a]PAF assays have not been carried out. See Table 1 for abbreviations.

DISCUSSION

Initial ethanol treatment of blood samples was shown to be superior to other extraction procedures as it effectively removes the bulk of protein and dark-colored pigments. As shown by use of [^{3}H]PAF, essentially all the radioactivity is recovered in the ethanol extracts. In addition, ethanol is known to inhibit PAF acetylhydrolase activity of erythrocytes (22). Also, plasma acetylhydrolase is completely inactivated under the present experimental conditions (personal observations). Although 95% of [^{3}H]PAF was recovered in the ethanol extracts, subsequent treatment of the precipitate with chloroform/methanol/water exhaustively extracted the blood samples. This second step alone yielded about 60% of the total PAF from blood samples of healthy individuals (see Table 1), thus indicating the presence of two distinct PAF pools in blood. The ethanol-extractable PAF, which we defined as "free" PAF, is probably loosely bound to serum proteins and lipoproteins (18). It was previously reported (18) that PAF bound to lipoproteins comigrated in HPLC with phosphatidylcholine, while in the present procedure "free" PAF eluted with a retention time equal to that of standard PAF. This would be expected as the silicic acid column chromatographic step and successive evaporation and redilution with polar solvents (chloroform/methanol) prior to HPLC purification would destroy proteolipid-type complexes. The PAF not extractable with ethanol, which we defined as "bound" PAF, may be the PAF strongly bound to lipoproteins and cellular structures and thus may not be quantitatively releasable with ethanol. "Bound" PAF was extracted only with a chloroform/methanol mixture from the ethanol precipitate. PAF co-migrated in HPLC with standard PAF. Initial use of chloroform/methanol/water for extraction of PAF is not recommended as it causes the formation of emulations and other technical problems. The second-step extraction with chloroform/methanol/water was omitted with urine samples since PAF was not detected in the ethanol precipitate. The effectiveness and simplicity of the extraction with ethanol has been well recognized by other investigators (17,18) even though

the presence of PAF in the ethanol precipitate had not been further investigated.

To find appropriate procedures for the purification of PAF, several approaches were tested. Addition of trichloroacetic acid at different concentrations to the ethanol extract failed to precipitate the residual proteins. Tests done with commercially available C_{18} and silica cartridges resulted in the incomplete removal of other lipids from the PAF fraction. Cartridge eluates containing PAF contained large amounts of other lipids, and especially SM, which caused insufficient resolution in the subsequent HPLC step as has also been reported by others (23). When the Sephadex G-25 purification was tested with [^{3}H]PAF as tracer (24), proteins were retained on the column, but [^{3}H]PAF was co-eluted with the total lipids. This led to a serious overload of the HPLC-column, causing insufficient resolution. Although the problem could be overcome by re-chromatography of the Sephadex-eluted lipid fraction on a silicic acid column (10 cm × 1 cm i.d.), which was eluted with methanol/water (2:1, vol/vol), the successive use of two columns made the method less suitable for routine analysis. To avoid the two column purification steps, the effectiveness of a single silicic acid column and the use of three different solvent mixtures containing methanol and water in different proportions was tested. Elution with methanol/water (2:1, vol/vol) caused complete overlap of PAF with nonlipid impurities; with methanol/water (1:1, vol/vol) PAF was eluted just following the nonlipid impurities; and elution with methanol/water (1:1.5, vol/vol) permitted the complete removal of impurities without any PAF losses, but required a significant volume of eluting solvents (220 mL).

Finally, the present method utilizes a silicic acid column and two successive eluting systems. The nonlipid impurities are removed with 45 mL of methanol/water (1:1.5, vol/vol) while PAF is eluted with the next 50 mL of methanol/water (2:1, vol/vol). The bulk of lipids is retained on the column, but can be removed with 40 mL of chloroform/methanol/water (1:2:0.8, by vol). Lipid–phosphorus analyses indicated that the PAF fraction consisted of only 1–2% of total phospholipids.

Several methods for the isolation and purification of PAF have previously been reported. According to Salari (19), proteins were precipitated by adjusting the pH to 5 with 1 M HCl, and PAF was then purified by XAD-2 solid-phase column chromatography. The protein precipitation step was found time-consuming and difficult for routine use. In addition, the protein precipitated with either ethanol/water or by ultracentrifugation would carry along significant amounts of PAF (18). Even more time-consuming and complex are the deproteinization and solid-phase column chromatographic purification steps that are used in PAF-RIA kits by Dupont (Platelet Activating Factor [^{125}I]radioimmunoassay kit NEK-062; NEN). Finally, the receptor-binding method (25) is not suitable for blood samples as has previously been reported (25).

HPLC is used in the present method for the final purification of PAF. The HPLC step has the advantage that PAF can be completely separated from other low-level phospholipids, including lysoPAF (21) (Fig. 1A) and various endogenous PAF inhibitors (26). As shown in Figures 1B and 1C, no significant HPLC peaks could be detected after silicic acid column chromatography. The necessity of the HPLC purification step for the complete purification of the blood samples was shown by comparative analyses, with and without HPLC purification, on ten and seven blood samples, respectively. The results shown in Table 1 indicate that omission of the HPLC purification step for blood extracts leads to PAF values that are almost two orders of magnitude lower than those obtained after HPLC purification. As shown in Table 1, "total" PAF ("free" plus "bound") ranged from 140–480 pg/mL of blood in the samples examined by the present method while 2.2–13.8 pg "total" PAF /mL were measured in blood samples examined without prior HPLC purification. HPLC purification is not required for the analysis of urine samples. The five urine samples examined by the present method were found to contain 1.2–4.0 pg PAF/mL urine, and four other samples gave similar values (4.0–13.8 pg PAF/mL) when examined without prior HPLC purification (Table 2).

The reproducibility of the method was tested by performing four analyses on each of three blood samples. The results shown in Table 3 indicate that the precision of the method is remarkable; standard deviations of the reproducability (SD overall) were less than 1% of the measured values of "free," "bound" and "total" PAF.

TABLE 3

Reproducibility of Native Platelet-Activating Factor (PAF) Assay on Selected Blood Samples[a]

m[b]	Subject number	"Free" PAF (pg/mL)	"Bound" PAF (pg/mL)	"Total" PAF (pg/mL)
1	12	140.3 ± 0.68	205.84 ± 2.5	346.15 ± 2.58
2	13	157.5 ± 1.28	220.5 ± 1.30	378.00 ± 0
3	14	138.2 ± 0.58	192.4 ± 0.94	330.65 ± 1.42
SD overall		0.90	1.72	1.70

[a]The blood samples selected were Nos. 12, 13 and 14 (Table 1); mean values ± SD; n = 4; SD overall = $\sqrt{\frac{\sum SD_i^2}{m}}$, where i = 1, 2, 3 . . . m, and m = 3.

[b]The number of samples.

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ARTICLE

Lipid Composition of the Pineal Organ from Rainbow Trout *(Oncorhynchus mykiss)*

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The lipid composition of the pineal organ from the rainbow trout (*Oncorhynchus mykiss*) was determined to establish whether the involvement of this organ in the control of circadian rhythms is reflected by specific adaptations of lipid composition. Lipid comprised 4.9% of the tissue wet weight and triacylglycerols were the major lipid class present (47% of total lipid). Phosphatidylcholine (PC) was the principal polar lipid, and smaller proportions of other phospholipids and cholesterol were also present. Plasmalogens contributed 11% of the ethanolamine glycerophospholipids (EGP). No cerebrosides were detected. The fatty acid composition of triacylglycerols was generally similar to that of total lipids in which saturated, monounsaturated and polyunsaturated fatty acids (PUFA) were present in almost equal proportions. Each of the polar lipid classes had a specific fatty acid composition. With the exception of phosphatidylinositol (PI), in which 20:4n-6 comprised 27.4% of the total fatty acids, 22:6n-3 was the principal PUFA in all lipid classes. The proportion of 20:5n-3 never exceeded 6.0% of the fatty acids in any lipid class. The predominant molecular species of PC were 16:0/22:6n-3 and 16:0/18:1, which accounted for 33.2 and 28.5%, respectively, of the total molecular species of this phospholipid. Phosphatidylethanolamine (PE) contained the highest level of di-22:6n-3 (13.0%) of any phospholipid. There was also 4.9% of this molecular species in phosphatidylserine (PS) and 4.1% in PC. In PE, the species 16:0/22:6, 18:1/22:6 and 18:0/22:6 totalled 45.1%, while in PS 18:0/22:6 accounted for 43.9% of the total molecular species. The most abundant molecular species of PI was 18:0/20:4n-6 (37.8%). The lipid composition of the pineal organ of trout, and particularly the molecular species composition of PI, is more similar to the composition of the retina than that of the brain.

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In fish, as in all vertebrates, the retina of the eye and the pineal organ of the brain are essential components of the circadian system that measures the period and phase of the daily light–dark cycle and ultimately controls rhythmic processes (1,2). In keeping with this role, the pineal organ of fish contains photoreceptor cells that display close cyto-functional analogies with the photoreceptors of the retina (3,4). The photoreceptor cells from both tissues translate the light–dark information into a neural output of excitatory neurotransmitter (5) and a neurohormonal output in the form of melatonin (3). In addition to being multimessenger cells, the photoreceptor cells of the pineal organ are also multieffectors and can transduce information supplied by other eternal (such as temperature) or internal (catechol-amines, adenosine, steroids) factors. Whereas retinal melatonin acts preferentially in an autocrine/paracrine manner, the melatonin secreted by the pineal contributes largely to the circulating levels and may be involved in the control of seasonal events, particularly reproduction, in vertebrates (3,6–8).

The structural phospholipids of the retina and brain of vertebrates, including fish, are known to contain high levels of the polyunsaturated fatty acid (PUFA) 22:6n-3 (9,10). Within the retina, the 22:6n-3 is apparently concentrated in the membranous outer segments of the photoreceptor rod cells (11), and these cells isolated from frog retina exhibit a selective uptake of 22:6n-3 *in vitro* (12). A requirement for 22:6n-3 in the visual process has been demonstrated in studies with newborn primates and preterm human infants, which have shown that the visual acuity is affected by deprivation of 22:6n-3 during postnatal development of the infant (13). Peroxidation of 22:6n-3 is one of the primary events observed in inherited or traumatically induced photoreceptor degeneration (9,14).

Di-22:6n-3 phospholipids are known to be major components of rod outer segment membranes in frog and rat (15,16). Recent analyses of the phospholipids of the brain and retina of trout and cod have shown that di-22:6n-3 molecular species are major constituents of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in these tissues and that this is particularly true of the retina, where the amounts are considerably higher than those found in terrestrial mammals (17,18). The importance of 22:6n-3 in the visual process of fish is also indicated by the observation that the proportion of di-22:6n-3 in the phospholipid of the developing eyes of herring larvae increases with age as the rods are recruited into the photoreceptor population (19). The results of these nutritional studies and the presence of large amounts of di-22:6 phospholipid in the photoreceptors of the retina suggest that 22:6n-3 has an essential and unique role in the primary events associated with the absorption and transduction of photons. This might be of crucial importance for the photoperiodic control of the production of messages, such as melatonin.

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Abbreviations: EGP, ethanolamine glycerophospholipids; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SM, sphingomyelin; TAG, triacylglycerols. Molecular species are abbreviated as follows: e.g., 16:0/22:6 PC is 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

By analogy with the retina, it is probable that 22:6n-3 is a major PUFA of the lipids in the photoreceptor cells of the fish pineal organ. To examine this hypothesis, we determined in the present study the detailed lipid composition of the trout pineal with particular attention to the PUFA content and molecular species of phospholipids. As far as we are aware, the detailed lipid composition of the pineal organ from fish has not been reported previously and only a limited amount of information is available on the lipids of the organ from mammals (20,21). Information gained from the analysis of the trout pineal organ is of basic importance as mammalian pinealocytes are accepted as being phylogenetically derived from the fish pineal photoreceptor cells (2).

MATERIALS AND METHODS

Fish and pineal organs. Rainbow trout (*Oncorhynchus mykiss*) of average weight 800 g were obtained from a commercial fish farm (Pisciculture Bellet, Angouleme, France) where they had been maintained under natural conditions of water temperature and photoperiod. One hundred fish were killed by decapitation. Pineal organs were removed immediately from the fish, frozen in liquid nitrogen and stored at –80°C until taken for analysis.

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).

Lipid extraction and analysis. After thawing, the pineal organs were weighed and transferred to a Teflon-in-glass homogenizer. The organs were homogenized in 37 mL of chloroform/methanol (2:1, vol/vol) essentially as described by Christie (22) to extract lipids. Solvent was removed under a stream of nitrogen and the resulting lipid extract desiccated overnight under vacuum in a preweighed tube. The tube and contents were then reweighed to obtain the weight of the lipid extract which was redissolved in chloroform/methanol (2:1, vol/vol) and stored under an atmosphere of nitrogen at –70°C between analyses.

To establish the lipid class composition, aliquots of lipid extract were subjected to high-performance thin-layer chromatography (HPTLC) alongside authentic standards using hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) as the developing solvent for the separation of neutral lipid classes and methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol) for the separation of polar lipids. To confirm which polar lipid classes were present, aliquots of total lipid were also subjected to two-dimensional HPTLC. The polar lipid developing solvent system described previously was used for development in the first dimension and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) was employed for development in the second dimension. Developed chromatograms were visualized with copper acetate in phosphoric acid (23). Lipid class composition was quantitated by double-development HPTLC coupled with scanning densitometry, as described elsewhere (24). Estimates of the relative amounts of the plasmalogen and the diacyl forms of ethanolamine glycerophospholipids (EGP) were obtained by acid hydrolysis of the isolated EGP *in situ* on an HPTLC plate followed by chromatography and quantitative phosphate staining as described by Bell and Dick (25).

For the analysis of fatty acid composition, individual lipid classes were separated by two-dimensional TLC on 20 × 20 cm glass plates coated with silica gel G 60 (0.25 mm thick) using the solvent systems described here. The separated classes were visualized by spraying the chromatogram with 0.1% 2′,7′-dichlorofluorescein in methanol containing 0.01% butylated hydroxytoluene and by viewing under ultraviolet light. Triacylglycerols (TAGs) were purified by redeveloping the chromatogram in the reverse direction of the second development using hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) after removal of the individual polar lipid classes. The fatty acids of the separated lipid classes were converted to their methyl esters on the adsorbent by acid-catalyzed transesterification (22). An aliquot of total lipid was also subjected to the same procedure. The resulting fatty acid methyl esters were purified by HPTLC and recovered from the adsorbent with hexane/diethyl ether (1:1, vol/vol).

Fatty acid methyl esters were analyzed on a Packard 439 gas chromatograph equipped with a fused silica capillary column (50 m × 0.22 mm i.d.) coated with FFAP phase (S.G.E., Milton Keynes, United Kingdom). Sample application was by on-column injection, and hydrogen was used as the carrier gas. During the course of an analysis, the oven temperature was programmed to increase from 50 to 225°C. Samples were also analyzed using an Omegawax 250 fused silica column (30 m × 0.25 mm i.d., Supelchem U.K. Ltd., Essex, United Kingdom) with the oven temperature programmed from 50 to 260°C. Fatty acid components were identified by reference to a well-characterized fish oil fatty acid mixture, and the unsaturated nature of components was confirmed by re-analysis of samples after catalytic hydrogenation over PtO_2. The separated components were quantitated using a recording integrator linked to the chromatograph.

Analysis of molecular species. A 500-μg portion of total lipid was separated into the component phospholipids by HPTLC alongside 20 μg of a cod retina total lipid standard using methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol) as the developing solvent. The standard spots and the edge of the bands of pineal organ lipids were visualized by exposure to iodine vapor, and the bands of adsorbent, containing PC, PS, phosphatidylinositol (PI) and EGP, were scraped from the plate. The phospholipids were hydrolyzed on the silica with phospholipase C using a two-phase system of 1 mL diethyl ether and 1 mL of 0.1 M sodium borate buffer, pH 7.5 at room tem-

perature under nitrogen for 2 h (26). At the end of the incubation period, 1,2-diradylglycerols were extracted, dried down under nitrogen and finally desiccated under vacuum for 1 h. 9-Anthroyl chloride was prepared from 9-anthracene carboxylic acid and oxalyl chloride as described by Goto *et al.* (27). The diradylglycerols were derivatized and purified by a modification of the method of Takamura and Kito (28) as described elsewhere (19). The 1-*O*-alk-1′-enyl-2-acyl derivatives were removed during the final HPTLC purification step. The 1,2-diacyl-3-anthrocyl-*sn*-glycerols were separated by HPLC at 19–20°C on an ODS column (25 × 0.46 cm, 5 μm particle size; Beckman Instruments U.K. Ltd., High Wycombe, Buckinghamshire, United Kingdom) using a Pye Unicam PU4010 pump (Pye Unicam Ltd., Cambridge, England) and two isocratic solvent systems, methanol/propan-2-ol (4:1, vol/vol) at a flow rate of 1.0 mL/min, and acetonitrile/propan-2-ol (7:3, vol/vol) at a flow rate of 1.0 mL/min as described by Takamura and Kito (28). Peaks were detected using a Waters 470 scanning fluorescence detector (Millipore UK Ltd., Edinburgh, Scotland) with excitation and emission wavelengths of 360 and 460 nm, respectively, and quantified using a Shimadzu CR3A recording integrator (Anachem, Luton, United Kingdom). Peaks were identified by their relative retention time using 16:0/22:6n-3 as a reference peak. Di-docosahexaenoylglycerol (di-22:6n-3; Nu-Chek-Prep, Elysian, MN) was also available for direct comparison of retention times, as were a range of samples of known composition from previous studies (17,18,29). Each sample was chromatographed three times in each solvent system and the standard deviations calculated. Where final peak areas were calculated by subtraction, the standard deviations of the contributing peaks were added to give the final error.

RESULTS

The lipid content and lipid class composition of trout pineal organ are presented in Table 1. Around 4.9% of the wet weight of the pineal organs was lipid, of which almost half (47%) was in the form of TAG. PC was the major polar lipid present (16.5% of total lipid), followed by EPG (9.9%). No choline plasmalogens were detected, whereas ethanolamine plasmalogens accounted for 11% of the total EPG fraction. PI and PS each accounted for less than 5% of the total lipid, and both phosphatidylglycerol (PG) and spingomyelin (SM) were present at less than 2%. No cardiolipin or cerebrosides were detected.

Palmitic acid (16:0) comprised 23.7% of the fatty acids in the total lipids and was the most abundant fatty acid in the pineal organ (Table 2). The monounsaturated 18:1n-9 and polyunsaturated 22:6n-3 accounted for 17.6 and 12.4%, respectively, of the total fatty acids. Overall, saturated, monounsaturated and polyunsaturated fatty acids accounted for similar proportions of the total lipid fatty acids. The fatty acid composition of TAGs (Table 2) was generally similar to that of the total lipid although the proportion of 18:2n-6 (12.8%) was notably higher, and that of 16:0 lower, than in total lipids.

TABLE 1

Lipids of Trout Pineal Organ: Lipid Class Composition[a]

Lipid class	% Total lipid
Cholesteryl esters	3.3 ± 0.2
Triacylglycerols	47.0 ± 2.1
Free fatty acids	3.4 ± 0.6
Cholesterol	8.7 ± 0.4
Diacylglycerols	1.5 ± 0.1
Ethanolamine glycerophospholipids	9.9 ± 0.6
Phosphatidylglycerol	1.4 ± 0.1
Phosphatidylinositol	3.2 ± 0.3
Phosphatidylserine	3.4 ± 0.3
Phosphatidylcholine	16.5 ± 0.8
Sphingomyelin	1.7 ± 0.1

[a]Values are means ± SD of three determinations.

TABLE 2

Fatty Acid Composition (wt%) of Total Lipid and Lipid Classes from Trout Pineal Organ[a]

Acyl chain	Total lipid	TAG	PC	EGP
14:0	2.9	3.5	0.6	0.2
15:0	0.9	0.3	0.3	0.9
16:ODMA	—	—	—	0.6
16:0	23.7	17.4	34.0	10.7
16:1n-9	—	—	0.6	—
16:1n-7	5.8	8.0	1.3	0.7
17:0	0.6	0.4	0.3	0.3
18:ODMA	—	—	—	0.4
18:1n-9DMA	—	—	—	2.2
18:1n-7DMA	—	—	—	0.7
18:0	7.2	4.2	5.4	10.2
18:1n-9	17.6	18.5	18.3	10.3
18:1n-7	4.0	3.7	2.4	3.9
18:2n-6	9.3	12.8	1.8	3.4
18:3n-3	1.2	2.0	—	0.3
20:1n-9	2.5	3.1	0.4	1.4
20:2n-6	0.6	1.2	0.4	0.9
20:3n-6	0.4	0.2	0.3	0.4
20:4n-6	1.6	0.5	2.0	7.2
20:3n-3	—	0.2	—	—
20:4n-3	0.9	1.4	0.3	0.6
20:5n-3	3.5	3.9	4.6	4.8
22:1n-11	1.7	2.0	—	—
22:1n-9	0.3	0.3	—	—
22:4n-6	0.1	0.3	—	—
22:5n-6	—	0.2	0.2	0.4
22:5n-3	1.2	1.8	1.3	2.2
22:6n-3	12.4	12.6	24.6	36.0
24:1n-9	0.7	0.1	—	0.4
Unidentified	0.9	1.4	0.9	0.7
Total saturated	35.3	25.8	40.6	23.3
Total monounsaturated	32.6	35.7	23.0	19.6
Total PUFA	31.2	37.1	35.5	56.2
Total n-3	19.2	21.9	30.8	43.9
Total n-6	12.0	15.2	4.7	12.3
n-3/n-6	1.60	1.44	6.55	3.57

[a]EGP, ethanolamine glycerophospholipids; PC, phosphatidylcholine; TAG, triacylglycerols; PUFA, polyunsaturated fatty acids; DMA, dimethylacetals.

TABLE 3

Fatty Acid Composition (wt%) of Lipid Classes from Trout Pineal Organ[a]

Acyl chain	PI	PS	PG	SM
14:0	0.3	0.3	0.8	0.6
15:0	0.4	0.7	0.3	0.4
16:0	14.0	3.0	10.7	19.2
16:1n-9	—	—	—	—
16:1n-7	—	9.3	17.0	0.5
17:0	0.7	0.4	0.5	0.6
18:0	33.8	38.2	5.4	9.5
18:1n-9	5.3	5.5	15.5	8.9
18:1n-7	1.3	2.6	10.2	1.9
18:2n-6	1.5	1.4	8.8	2.2
18:3n-3	0.1	0.4	0.9	—
20:1n-9	0.2	1.2	1.4	0.4
20:2n-6	0.2	0.4	1.1	1.0
20:3n-6	0.3	—	1.0	1.0
20:4n-6	27.4	1.0	1.8	0.5
20:3n-3	—	—	—	—
20:4n-3	0.1	—	0.5	0.2
20:5n-3	5.7	1.3	1.6	0.9
22:1n-11	—	—	—	—
22:1n-9	—	—	—	0.6
22:4n-6	0.5	0.9	0.9	—
22:5n-6	—	0.4	—	—
22:5n-3	0.4	3.0	0.7	0.2
22:6n-3	7.0	29.4	19.8	2.9
24:0	—	—	—	0.8
24:1n-9	0.4	—	—	45.9
Unidentified	0.4	0.6	1.1	1.3
Total saturated	49.2	42.6	17.7	31.6
Total monounsaturated	7.2	18.6	44.1	58.2
Total PUFA	43.2	38.2	37.1	8.9
Total n-3	13.3	34.1	23.5	4.2
Total n-6	29.9	4.1	13.6	4.7
n-3/n-6	0.45	8.32	1.73	0.89

[a]PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PUFA, polyunsaturated fatty acids.

Each of the polar lipid classes had a specific fatty acid composition. In PC, 16:0 and 22:6n-3 were the principal components and together accounted for nearly 60% of the total fatty acids present in this phospholipid (Table 2). In contrast, 16:0 comprised only 10.7% of the fatty acids in EGP, whereas the level of 22:6n-3 (36%) was the highest observed in any lipid class. As a consequence, the total content of PUFA in EGP (56.2%) was the highest of all lipid classes. Only the EGP fraction produced dimethyl acetals by transmethylation of 1-*O*-alk-1′-enyl linked ether chains.

PI was unique among the lipid classes in that 20:4n-6 was a major component and accounted for 27.4% of the fatty acids, whereas 22:6n-3 comprised only 7.0% (Table 3). As a consequence, the overall ratio of n-3 to n-6 PUFA in PI was the lowest of any lipid class. The saturated fatty acid 18:0 was also a major fatty acid in PI. PS was characterized by a high content of 18:0 and 22:6n-3, coupled with a low content of 16:0. PG was rich in monounsaturated fatty acids and PUFA which comprised 44.1 and 37.1%, respectively, of the total fatty acids in this phospholipid (Table 3). The levels of 16:1n-7 and 18:1n-7 (17.0 and 10.2%) in PG were higher than in any other lipid class, and, of all the phospholipids PG had the highest content of 18:2n-6. In SM more than half (58.2%) of the fatty acids were monounsaturated, mainly due to the presence of a very high proportion of 24:1 (45.9%). PUFA comprised only 8.9% of the SM fatty acids (Table 3).

In all lipid classes, 20:5n-3 was present in small amounts and never exceeded 6.0% of the component fatty acids. The long chain monoenoic fatty acid 22:1n-11 observed in total lipid was concentrated in TAG, where it accounted for around 2% of the fatty acids. Of all the lipid classes, TAG also contained the highest level of 14:0.

The principal molecular species of PC, PE, PS and PI are presented in Table 4. Two molecular species predominated in PC, namely 16:0/22:6n-3 and 16:0/18:1, which accounted for 33.2 and 28.5%, respectively, of the total molecular species of this phospholipid. Di-PUFA, disaturated and di-monounsaturated species each comprised less than 6% of the total PC and the content of monounsaturated-PUFA species totalled 4.9%, within which 18:1/22:6n-3 was the major component. Molecular species containing 22:6n-3 were particularly abundant in PE. Di-22:6n-3 comprised 13.0% of the total, and 16:0/22:6 and 18:1/22:6 were both present at levels of more than 15%. In PS, 18:0/22:6n-3 accounted for almost half (43.9%) of the total molecular species, and 16:0/22:6n-3 was the only other species present at a level of greater than 10%. The most abundant molecular species of PI was 18:0/20:4n-6 which accounted for 37.8%. Another species containing 20:4n-6, 16:0/20:4n-6, comprised 14.0% of the molecular species of PI and 18:0/20:5n-3 accounted for 10.8%, the highest level for a species containing 20:5n-3 observed in any of the phospholipids examined.

DISCUSSION

Although the pineal organ is an adjunct of the brain, it is known to have evolved from a well-differentiated photoreceptive organ that is frequently considered to be a functional third eye in lower vertebrates (3). In fact, to date, the properties established for the pineal photoreceptors have been extended to the retinal photoreceptors and *vice versa* (3). The major difference between the pineal and retina relates to the neuronal organization, which is simple in the pineal but complex in the retina. Thus, pineal photoreceptors make contact with second order neurons that send their axons to brain centers. Retinal photoreceptors, on the other hand, are in contact with bipolar cells that, in turn, communicate with ganglion cells and numerous interneurons that are present (horizontal, amacrine, interpexiform cells). Consequently, the ratio of photoreceptors to other neurons is much higher in the pineal than in the retina, and the lipid composition of the pineal organ can be expected to reflect that of the photoreceptor cells.

TABLE 4

Molecular Species of Phospholipids from Trout Pineal Organ[a]

Species	PC	PE	PS	PI
Di-PUFA				
20:5/22:6	0.6 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	–
22:6/22:6	4.1 ± 0.2	13.0 ± 0.3	4.9 ± 0.0	0.3 ± 0.0
22:5/22:6	1.0 ± 0.1	4.5 ± 0.3	2.4 ± 0.1	0.1 ± 0.0
Saturated/PUFA				
14:0/22:6	1.2 ± 0.1	3.8 ± 0.1	0.7 ± 0.2	0.2 ± 0.1
16:0/20:5	6.7 ± 0.4	4.2 ± 0.1	1.8 ± 0.3	4.1 ± 0.2
16:0/22:6	33.2 ± 1.6	20.1 ± 0.1	15.0 ± 0.2	10.3 ± 0.5
16:0/20:4	1.5 ± 0.2	2.4 ± 0.1	1.8 ± 0.1	14.0 ± 0.3
16:0/22:5	2.1 ± 0.3	1.9 ± 0.1	1.1 ± 0.1	0.6 ± 0.2
18:0/20:5	1.2 ± 0.2	3.7 ± 0.1	3.7 ± 0.3	10.8 ± 0.1
18:0/22:6	3.7 ± 0.2	9.6 ± 0.3	43.9 ± 0.2	7.1 ± 0.5
18:0/20:4	0.5 ± 0.1	2.7 ± 0.1	0.6 ± 0.1	37.8 ± 1.0
18:0/22:5	1.5 ± 0.2	0.6 ± 0.2	4.7 ± 0.1	0.4 ± 0.1
Monounsaturated/PUFA				
16:1/22:6	0.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.2 ± 0.0
18:1/20:5	0.9 ± 0.1	3.7 ± 0.1	–	0.7 ± 0.0
18:1/22:6	2.9 ± 1.7	15.4 ± 0.4	6.3 ± 1.0	0.6 ± 0.5
18:1/20:4	–	2.6 ± 0.1	1.1 ± 0.1	4.1 ± 0.2
18:1/22:5	0.6 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	–
Di-saturated				
16:0/16:0	0.9 ± 0.1	0.1 ± 0.0	1.1 ± 0.1	–
Saturated/monounsaturated				
16:0/18:1	28.5 ± 1.0	1.2 ± 0.1	2.8 ± 0.0	6.9 ± 0.3
18:0/18:1	2.5 ± 0.1	0.2 ± 0.1	0.9 ± 0.0	0.3 ± 0.0
Di-monounsaturated				
18:1/18:1	0.8 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	0.6 ± 0.0
20:1/18:1	–	–	0.3 ± 0.0	–

[a]Values are mol% and are means of triplicate determinations ± 1 SD. It was assumed that the most saturated fatty acids were located on the *sn*-1 position. Molecular species containing minor fatty acids or fatty acid isomers were not resolved from the major components. Molecular species containing 18:2n-6 co-elute with those containing 22:5n-3 and 22:5n-6. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids.

The lipid content of the trout pineal (4.9% of wet weight) is almost exactly halfway between that found for the brain and retina (6.8 and 3.1%, respectively) of the same species (10). However, the level of TAG in the lipid of the pineal (47%) is considerably higher than the 8.4 and 30% found in the lipid of the brain and retina, but is nevertheless closer to the latter. The levels of TAG in brains and retina of trout are known to be higher than in the same tissues from cod (10). An influencing factor may be the fact that the trout examined were farmed fish. It is well known that farmed fish, including trout, have higher lipid contents in their flesh than their wild counterparts (30). Whether the lipid class content of neural tissues differs between wild and farmed fish or whether high TAG levels are a specific feature of the lipids of trout neural tissues remains to be established.

A notable feature of the brain of fish and animals in general is the high proportion of EGP in the total lipid (10,31). In both cod and trout, the EGP/PC ratio in brain lipid is approximately 1:1 (10). The level of EGP in the pineal lipid is notably lower than that of PC, the EGP/PC ratio being 0.6:1. This value is closer to the 0.7:1 observed for the ratio of these two phospholipids in the retina of both trout and cod (10). The ethanolamine plasmalogen content of the pineal is much lower than that of fish brain (36–38% of EGP) (25) and closer to that of retina (<5% of EGP; Bell, M.V., unpublished data). The absence of cerebrosides in the lipids of the pineal is also more typical of retina than brain, as is the low amount of ethanolamine plasmalogens (31). The absence of cardiolipin in significant amounts in pineal lipids is unusual as this lipid class is a common component of mitochondrial membranes and has been found in lipid extracted from both fish retina and brain (10).

The total lipid and component lipid classes of the pineal organs, with the exception of SM, were characterized by high contents of PUFA. Nevertheless, the content of PUFA in the total lipid of trout organ (31.2%) is lower than the corresponding values of 41.4 and 40.6% reported for the brain and retina, respectively, of the same species (10), and is considerably less than the PUFA content (43.6%) of total lipid from rat pineal (20). The distribution of fatty acids among the various lipid classes conforms to well established patterns. For example, the long-chain monoenoic fatty acid 22:1n-11 occurred only in TAG. The absence of this fatty acid, which

originates in calanoid zooplankton, is a common feature of fish phospholipids (32). The PUFA 18:2n-6 was also confined to TAG. It is notable that the total lipid of pineal gland contains higher levels of this fatty acid than brain in the rat (20), although it remains to be established whether the 18:2n-6 is specifically associated with TAG. The presence of high levels of 16:0 in PC is a characteristic feature of this phospholipid, including that extracted from trout brain and retina (10) and bovine pineal organ (21). The EGP of neural tissues are known to be specifically enriched in 22:6n-3 (31), and this was also a notable feature observed in the present study of the EGP from the trout pineal. The value for 22:6n-3 in EGP (36%) is slightly higher than the content in EGP from trout brain (34%), but less than that of the retina EGP (46.3%) of the same species (10). Interestingly, the 22:6n-3 contents of PC, PI and PS found here in the pineal are all intermediate between those found in the brain and in the retina.

The PI of the pineal organ conformed to the well established pattern in fish tissues, whereby PI has a higher content of 20:4n-6 than other phospholipids and is consequently characterized by a low ratio of n-3 to n-6 PUFA (32). The actual content of 20:4n-6 in the pineal PI (27.4% of total fatty acids) is markedly higher than that reported for trout brain (10.2%) and retina (14.8%) and for the same tissues of cod (4.4 and 12.5%, respectively) (10). The results suggest that in terms of the 20:4n-6 content of PI the pineal exceeds retina which, in turn, exceeds brain. In the rat, the level of 20:4n-6 in the total lipids from the pineal organ is also higher than that found in brain lipids (20). This is consistent with the idea according to which phototransduction might activate phospholipase A_2 with the subsequent formation of eicosanoids (33). An involvement of eicosanoids derived from 20:4n-6 in the light-dependent control of melatonin production is therefore indicated.

Consistent with the overall fatty acid composition of EGP, PE contained the highest level (13.0%) of di-22:6 molecular species of the phospholipids examined. However, the value was less than that observed previously in trout brain (14.9%), and considerably less than that in trout retina (41.3%) (17). Likewise, di-22:6 molecular species of PC and PS were less abundant in the pineal than the retina, and only pineal PC had a higher di-22:6 content than the corresponding phospholipids from brain. The other molecular species were all as expected, with 16:0/22:6 and 16:0/18:1 dominating PC, 16:0/22:6, 18:1/22:6 and 18:0/22:6 each comprising 9.6–20.1% of PE, and 18:0/22:6 being the predominant PS species. In contrast, PI from fish is known to be relatively impoverished with respect to 22:6n-3 containing molecular species, while C_{20} PUFA species are abundant (17,18). In trout, retinal PI was predominantly 18:0/20:4n-6 (40.1%) and brain PI 18:0/20:5n-3 (42.3%) (17). This tissue specificity in the molecular species composition of PI was confirmed in cod, in which 18:0/20:4n-6 was the predominant species in liver and roe (36.7 and 49.1%, respectively) and 18:1/20:4n-6 was the next most abundant (18,29). In brain, 18:0/20:5n-3 and 18:1/20:5n-3 comprised over half the PI (18) with arachidonyl species totalling only 15.3%, whereas in retina 16:0/22:6, 18:0/20:4, 18:0/22:6 and 18:0/20:5 were the most abundant species, in that order, totalling 84.5% of PI (18).

The PI from trout pineal organ thus closely resembles that of trout retina with 18:0/20:4n-6 predominant. The 18:0/20:5n-3 species so characteristic of PI from trout and cod brain comprised only 10.8% of trout pineal PI. Thus, although it is situated adjacent to the brain and is very closely associated with the brain, the pineal is almost identical to retina in terms of PI composition. Furthermore, no 18:1/24:1 was found in the PC of the pineal organ, whereas this molecular species comprises between 9 and 13% of fish brain PC (17,18).

Although the total lipid of the trout pineal organ has a lower content of 22:6n-3 than that found in brain or retina, the level is nevertheless still higher than that usually observed in other non-neural tissues, such as liver or muscle (32). Furthermore, the 22:6n-3 content of individual phospholipids is similar to that found in the retina and brain. Consequently, the lipid composition of the pineal organ displays features typical of nervous tissues.

Di-22:6n-3 molecular species may be unique to photoreceptor membranes as these are abundant in rod outer segments of many animal species and have recently also been found, albeit in smaller amounts, in the all-cone retina of larval herring (19). The outer segments of the pineal photoreceptors, which correspond to cone-like cells, are not as well developed compared to those of the retinal photoreceptor (3), and the overall amount of outer segment membrane per photoreceptor cell is probably considerably less in the pineal than in the retina. This might explain why the high levels of di-22:6n-3 molecular species which characterize the PC, and especially PS and PE, of trout and cod retina (17,18) were not observed in the trout pineal.

In conclusion, the present study demonstrates for the first time that, in terms of lipid composition, the pineal resembles the retina more closely than the brain or other tissues. Photoreceptor membranes can now be isolated from a population of purified pineal cells (34) and analysis of their component lipids may disclose specific adaptations in lipid composition. This opens interesting perspectives for the study of the light-dependent signal production by these cells.

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Comparison of Body Weight and Adipose Tissue in Male C57Bl/6J Mice Fed Diets with and without *trans* Fatty Acids

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The effect of a diet containing *trans*-fatty acids (*t*FA) on the fatty acid composition and fat accumulation in adipose tissue was investigated in mice. Male C57Bl/6J mice were fed Control or Trans Diets that were similar, except that 50% of the 18:1, which was all *cis* in the Control Diet, was replaced by *t*FA in the Trans Diet. At selected ages, body weight, epididymal fat pad weight, perirenal fat yield, adipose tissue cellularity and fatty acid composition were examined. Over the time period studied (2–24 mon), the proportion of 18:0 and 16:0 tended to decrease while *cis*-18:1 levels increased. Compared to the Control Diet, the Trans Diet resulted in adipose tissue lipids with higher percentages of 14:0 and 18:2n-6 and lower percentages of *cis*-18:1 and 20:4n-6. In polar lipids, *t*FA replaced saturated fatty acids, whereas *t*FA replaced *cis*-18:1 in the nonpolar lipids. Body weights at 16 and 24 mon of age and epididymal fat pad weights at 8–24 mon of age were lower in mice fed the Trans Diet as compared to those fed the Control Diet. At the ages studied, the Trans Diet also resulted in lower values for perirenal fat weights, triacylglycerol to polar lipid ratios, and adipose cell size. The data suggest that chronic consumption of *t*FA affects lipid metabolism and results in decreased fat accumulation in murine adipose tissue.

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Trans fatty acids (*t*FA) are not products of *de novo* fatty acid synthesis in animal cells. Nevertheless, *t*FA are incorporated into most animal and human tissues (1–7). The ultimate metabolic fate and the physiological effects of *t*FA have not been fully characterized. However, in light of recent findings (8–10), it has become increasingly clear that the physiological properties of *t*FA may be substantially different from those of their *cis*-monounsaturated counterparts.

Earlier studies that examined the biological effects of *t*FA have not always included adequate control diets. When comparisons are made between diets that differ not only in *t*FA, but also in the proportions of other fatty acids, caution must be used not to attribute differences solely to the presence of *t*FA. In our laboratory (11) we attempted to blend dietary fats by mixing various edible oils so that the diets differed only in the ratio of 18:1*t* to 18:1*c* fatty acids; by doing so, differences observed are more likely related to differences in *t*FA in the diets.

Previous work in our laboratory with properly matched diets had demonstrated that diets containing *t*FA can depress the percentage of fat in mouse milk (11). As the major lipids synthesized in mammary tissue are triacylglycerols, and the major products of lipid metabolism in adipose tissue are also triacylglycerols, we initiated studies using epididymal fat pads of male C57Bl/6J mice to determine whether lipid metabolism in adipose tissue is also affected by dietary *t*FA. As we pursued these studies, it became clear that diets containing *t*FA affected the fat depot weights and the ratios of triacylglycerol to phospholipid in epididymal fat pads. These findings led us to explore in further detail age-related changes in adipose depots of mice fed diets containing *t*FA compared to mice fed a control fat. the results of these studies are described here.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, all solvents used were reagent grade or better. Chloroform, methanol, methylene chloride, hexane, diethyl ether and petroleum ether were obtained from J.T. Baker (Phillipsburg, NJ); isooctane used as solvent for glass capillary gas–liquid chromatography (GCGLC) was obtained from Burdick and Jackson (Muskegan, MI). Quadrex (New Haven, CT) and Supelco (Bellefonte, PA) were the sources of the fused silica GCGLC columns coated with SP2340 and SP2560, respectively. Standard fatty acid methyl ester (FAME) mixtures that were used for calibration were from Nu-Check-Prep (Elysian, MN). Silica Gel G used for thin-layer chromatography (TLC) of FAME and for the separation of nonpolar from polar lipids was a product of E. Merck (Elmsford, NY). Silica Gel LK 5D TLC plates (20 × 20 cm, 250 μm) used in TLC spectrodensitometry were purchased from Whatman (Clifton, NJ).

Some of the dietary components [casein, alphacel, American Institute of Nutrition (AIN) (Bethesda, MD) mineral mix-76, AIN vitamin mix-76A, dl-methionine and choline bitartrate] were obtained from ICN Nutritional Biochemicals (Cleveland, OH). Sucrose was from Mazzeo and Sons (Hyattsville, MD). Commercial frying shortening was from Auth Brothers (Washington, DC), cocoa butter was from Wilbur Chocolate Co. (Lititz, PA) and Pope olive oil from Continental Smelkinson (Jessup, MD). Mazola corn oil and soybean oil were purchased from local Giant Food supermarkets.

Diets. The diet ingredients used are given in Table 1. Except for the fat source, the two diets were identical. The Trans Diet contained a mixture of shortening, corn oil and soybean oil, while the source of fat in the Control Diet was a mixture of olive oil, corn oil and cocoa butter. The fats were blended to yield fatty acid compositions (Table 2) that were essentially the same except that ≈50% of the 18:1, which was all *cis* in the Control Diet, was replaced by *t*FA in the Trans Diet. Both diets contained ≈6 cal% linoleic acid and were in compliance with AIN rodent nutritive guidelines (12).

Animals. Mice used in these studies were housed in our animal facility under controlled conditions of

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Abbreviations: AIN, American Institute of Nutrition; FAME, fatty acid methyl esters; GCGLC, glass capillary gas–liquid chromatography; *t*FA, *trans*-fatty acids; TLC, thin-layer chromatography.

TABLE 1

Diet Components in the Control and Trans Diets

Ingredients[a]	g/kg of diet dry weight
Sucrose	590
Casein	200
Fat[b,c]	100
Alphacel	50
AIN[d] mineral mix	40
AIN vitamin mix	15
Methionine	3.0
Choline bitartrate	2.0

[a]Ingredient sources are listed in Materials section.

[b]Edible oil content of the diets were: Control Diet, olive oil, 57%; corn oil, 31%; and cocoa butter, 12%; Trans Diet, shortening, 57.15%; corn oil, 33.33%; and soybean oil, 9.52%.

[c]See Table 2 for fatty acid composition of the two diets.

[d]American Institute of Nutrition (Bethesda, MD).

temperature (≈23°C), humidity (≈60%) and lighting (12 h day/night cycle) and were provided food and water *ad libitum*. Handling and care of mice were done strictly according to guidelines of the Animal Care and Use Committee of the University of Maryland (College Park, MD).

Weanling C57Bl/6J mice, obtained from Jackson Laboratories (Bar Harbor, ME), were randomly assigned as mating pairs and fed one of the two diets described above. Male offspring were weaned at three weeks of age, assigned to the same diet as their respective parents and used at selected ages in the various studies. Selection of animals used in all experiments was done randomly such that for each experiment the animals selected from a particular diet comprised individuals pooled from different litters.

For each of the following studies, the number of animals from each diet used at each age is given in parentheses. Mice were examined at 2, 4, 6, 8, 12, 15, 16 and 24 mon of age to measure epididymal fat pad weight (n = 4–6), body weight (n = 4–5) and epididymal fat pad fatty acid composition (n = 3). Studies on the amount of perirenal fat (n = 20) were done using eight-month-old mice, investigations on triacylglycerol/polar lipid ratios (n = 3–5) were done on ten-month-old mice, and studies on adipose cell size and number (n = 3) were done on 24-month-old mice. Mice at eight months of age were used for studies on the fatty acid composition of the nonpolar and of the polar lipids of epididymal fat pads (n = 3, pooled samples of four mice each).

Adipose tissue weights and body weights. Body weights were recorded prior to killing the animals with CO_2. Epididymal fat pads and, in some cases, perirenal fat tissue, were excised and weighed.

Lipid extraction and preparation of FAME. Lipids were extracted essentially as described by Folch *et al.* (13). Tissues were minced and homogenized in 19 vol of chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene as an antioxidant. The crude lipid extract was separated into an organic and an aqueous phase by the addition of an appropriate amount of 0.58% NaCl. The lipid in the lower organic phase was concentrated, and aliquots (≈25 mg) were transesterified with methanolic HCl for 16 h at 80°C. The resulting FAME were purified on Silica Gel G TLC plates. The extraction, esterification and purification procedures have been described previously (14).

Separation and analysis of polar and nonpolar lipid. Pooled lipids from epididymal fat pads of four mice were applied (≈2 g/plate) to 20 × 20 cm TLC plates coated with a 0.5-mm layer of Silica Gel G. The plates were developed three times to the top with diethyl ether. After spraying with dichlorofluorescein, the polar and nonpolar lipid bands were visible under ultraviolet light. Then the polar lipid area and a longitudinal portion of the much larger, nonpolar lipid area were scraped into test tubes. Sufficient anhydrous methanolic HCl (2 mL) and methylene chloride (3 mL) were added to cover the silica; the tubes were vigorously vortexed and incubated at 80°C for 16 h to prepare FAME. The transesterified lipids were extracted with hexane, and the purified FAME were isolated using silica gel TLC as described (14).

GCGLC. Purified FAME were concentrated under a stream of nitrogen and dissolved in isooctane for analysis by GCGLC. Analyses were conducted on 5830, 5840 or 5880 Hewlett-Packard gas chromatographs (Avondale, PA) equipped with flame-ionization detectors and capillary split injection ports. A glass column (15 m × 0.25 mm) coated with SP2340 was used in some of the work, but most of the analyses were conducted on fused silica columns (25 m × 0.25 mm coated with SP-2340 or 100 m × 0.25 mm coated with SP-2560). Column conditions and other parameters were essentially as described previously (14).

Authentic mixtures of FAME were used to identify components and to obtain appropriate response factors. When the shorter columns were used, correction factors were required as *trans*-octadecenoates (18:1*t*) were underestimated and *cis*-octadecenoates (18:1*c*) were overestimated. To establish the correction factors, argentation TLC was used to isolate the 18:1*t* and 18:1*c* fractions from the Trans Diet fat as described previously (14). After adding internal standards to the separated 18:1 fractions, a series of mixtures were prepared, the mixtures were analyzed on the 15- and 25-m columns, appropriate correction factors were determined, and an equation relating the correction factors and the observed percentage of the 18:1*t* in the total 18:1 was developed

TABLE 2

Fatty Acid Composition of Control and Trans Diets[a]

	Diet	
Fatty acid	Control	Trans
16:0	13.4 (0.5)	10.9 (0.7)
18:0	6.5 (0.4)	9.8 (0.2)
18:1*t*	nd	25.5 (0.3)
18:1*c*	51.1 (0.7)	25.4 (0.6)
18:2n-6	26.1 (0.6)	25.6 (0.8)
18:3n-3	0.9 (0.1)	0.9 (0.1)

[a]Means as weight percentage of the total fatty acid methyl esters for ten independent determinations with the standard deviation given in parentheses; nd, not detected.

for each column essentially as described previously (14). The equation was then used to obtain the correction factor for the 18:1*t* fraction in each sample analyzed on that specific column. Because the sum of the 18:1*c* and 18:1*t* before and after correction should be identical, corresponding correction factors for the 18:1*c* fraction in each sample were obtained algebraically.

Triacylglycerol/polar lipid ratio. Relative levels of triacylglycerols to polar lipids were determined using TLC densitometry. Duplicates of total lipid extracts for each sample were spotted on Whatman silica gel LK 5D 20 × 20 cm, 250-μm plates. Graded amounts of triacylglycerol (previously isolated from mouse epididymal fat pads) and phospholipid (egg phosphatidylcholine, ≈99% purity; Sigma Chemical Co., St. Louis, MO) were spotted on separate lanes to obtain a standard curve for the nonpolar and polar lipid fractions. The plates were developed in hexane/diethyl ether/formic acid (80:20:2, by vol), allowed to air-dry, dipped momentarily in 2% sulfuric acid, and charred at 170°C, essentially as described by Bitman *et al.* (15). The plates were then scanned using a Shoeffel Instruments Model SD3000 spectrodensitometer (Westwood, NJ) equipped with a Hewlett-Packard 3390A Reporting Integrator.

Adipose tissue cellularity. Epididymal fat pads were excised and immediately transferred to normal saline at 37°C. Pools of proximal, distal and medial fragments totalling less than 100 mg per fat pad were used for osmium fixation as described by Hirsch and Gallian (16).

Statistics. The results of most experiments were analyzed using the *t*-test or analysis of variance. Regression analysis was used to test the inverse relationship between body weights and adipose tissue 18:1*t* content.

RESULTS

Body weights and epididymal fat pad weights for mice on the two diets are given in Figure 1. The general patterns obtained over the time period examined were similar. Both body weight and epididymal fat pad weight increased up to 15 or 16 mon of age and then appeared to decline sharply. Compared to mice fed the Control Diet, mice on the Trans Diet had lower body weights ($P < 0.05$) at the two oldest ages examined (16 and 24 mon) and lower epididymal fat pad weights ($P < 0.05$) at all ages examined between 8 and 24 mon. When the body weights of the mice on the Trans Diet were plotted against their epididymal fat 18:1*t* content (Fig. 2), a significant inverse correlation ($r = 0.68$, $P < 0.002$) was observed.

Diet related differences were also evident when the amount of perirenal fat at eight months of age (Fig. 3) was examined and when epididymal fat pad triacylglycerol to polar lipid ratios were studied at ten months of age (Fig. 4). The yield of perirenal fat from mice fed the Trans Diet was only about half of that isolated from mice fed the Control Diet ($P < 0.0004$), and the triacylglycerol to polar lipid ratio in epididymal fat pad lipids of mice fed the Trans Diet was about one-fourth ($P < 0.025$) of that observed in mice fed the Control Diet. These differences were apparently related primarily to changes in cell size rather than cell number, as studies of adipose cellularity at 24 mon of age (data not shown) revealed that the average size of epididymal fat pad cells isolated from mice fed the Trans Diet was about 25% less ($P < 0.0001$) than that of cells isolated from mice fed the Control Diet. Differences in cell numbers were not statistically significant.

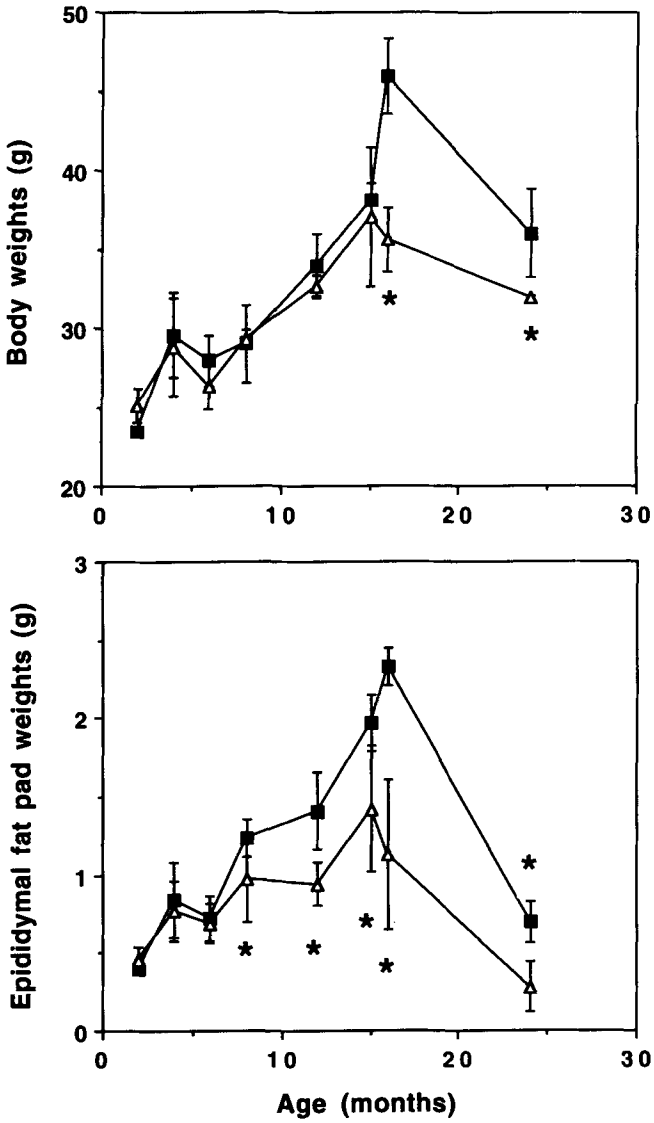

FIG. 1. Top panel depicts body weights of mice on Control Diet or Trans Diet at various ages. Bottom panel depicts epididymal fat pad weights of mice on the two diets at various ages. Squares are data for mice on the Control Diet; the triangles are data for mice on the Trans Diet. Values are means ± SEM of three mice per diet at each age group; * indicates that values from mice fed the Trans Diet were significantly smaller ($P < 0.05$) than those fed the Control Diet.

The effects of diet and age on fatty acid composition of epididymal fat pads in mice are depicted in Table 3. Age-related trends include a decrease in the proportion of 16:0 and possibly 18:0 as well as an increase in the proportion of 18:1*c* with age. Compared to the Control Diet, the Trans Diet tended to result in higher proportions of 14:0 and 18:2n-6 and lower proportions of 18:0, 18:1*c*

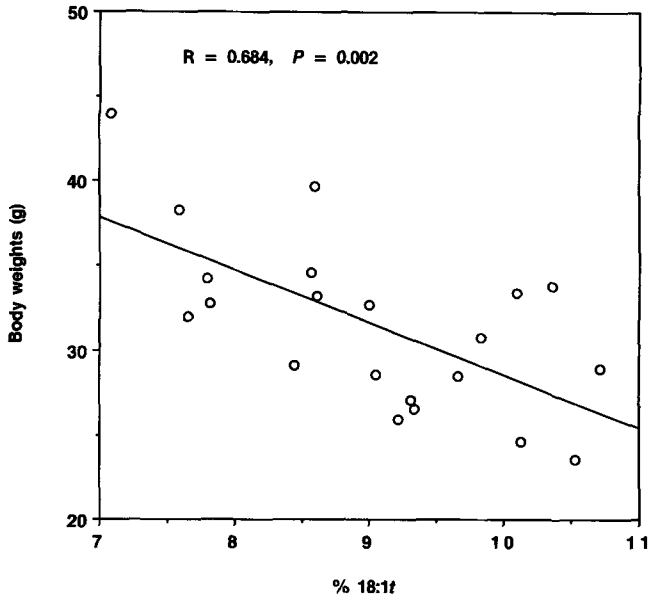

FIG. 2. Relationship between % *trans* 18:1 in epididymal adipose tissue and mouse body weights. Values are for 21 mice on the Trans Diet.

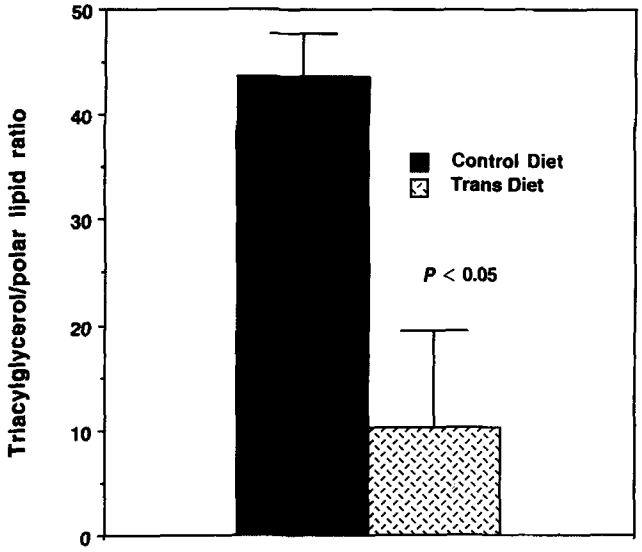

FIG. 4. Triacylglycerol/polar lipid ratios in epididymal adipose tissue as estimated by thin-layer chromatography densitometry. Values are means ± SEM of ratios obtained from three mice on each diet.

and 20:4n-6. Relative to the percentages present in the diets, proportionately more 18:1*c* was present in epididymal fat pads isolated from mice fed the Trans Diet.

The fatty acid compositions of polar and nonpolar lipids of epididymal fat pads isolated from eight-month-old mice fed the Control or Trans Diet are summarized in Tables 4 and 5. In the polar lipids (Table 4), the incorporation of 18:1*t* was accompanied by a reduction in the proportion of 16:0 and 18:0, but not of 18:1*c*. By contrast, in the nonpolar lipids (Table 5), the Trans Diet caused significantly lower proportions of 18:1*c*, but not of 16:0 and 18:0. In the polar lipids, but not in the nonpolar lipids, the value for the sum of 18:1*t* plus saturated fatty acids in tissue isolated from mice fed the Trans Diet was close to the value for total saturated fatty acids in tissues isolated from mice fed the Control Diet.

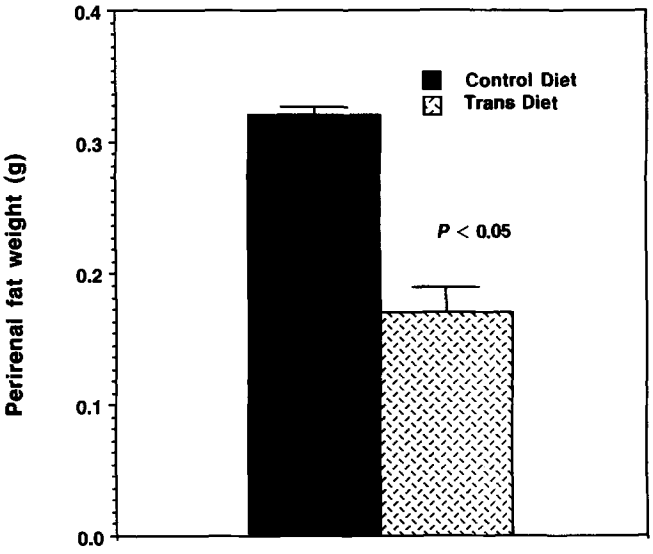

FIG. 3. Perirenal fat weights of eight-month-old mice. Values are means ± SEM from three mice on each diet.

DISCUSSION

The major observations made in the present study were the effects of diet on fat accumulation in male mice. Compared to mice on the Control Diet, mice fed the Trans Diet for at least eight months had lower epididymal fat pad weights, a lower triacylglycerol/polar lipid ratio in lipids isolated from epididymal fat pads, a lower perirenal fat weight and a smaller epididymal fat cell size. Also, at the two oldest ages examined, the mice on the Trans Diet had lower body weights than the mice fed the Control Diet. These findings suggest that compared to the Control Diet, chronic consumption of the Trans Diet resulted in decreased fat accumulation in different adipose depots. Recent studies (17) with 3T3-L1 cells, a subclone of Swiss mouse embryo 3T3 fibroblasts, suggest that this phenomenon may be common for adipose tissue in mice.

These data are in conflict with those of Ostlund-Linqvist *et al.* (18), who reported that adipocytes from rats fed *t*FA had significantly larger adipose cells as compared to those fed a control diet. Species-specific differences with regard to the effects of dietary fats or the extent of feeding may account for the conflicting data. In the earlier study (18), four-week-old rats were fed for only two months. However, differences in the methodology used to examine adipose cell size may also be responsible. Adipocytes are very fragile under *ex vivo* conditions, and the possibility of cell breakage cannot be ruled out. In addition, differences in fatty acid composi-

TABLE 3

Fatty Acid Composition of Epididymal Adipose Tisse in Mice Fed Control and Trans Diets for 2–24 Months[a]

Fatty acid	Diet	Age (months) 2	4	6	8	12	15	16	24	SEM
14:0	Control	0.8	0.8	0.6	0.6	0.7	0.7	0.7	0.7	0.04
	Trans	1.1	0.9	1.0[b]	1.0[b]	0.7	0.9	0.8	1.1	0.04
16:0	Control	17.1	16.6	14.9	14.7	13.9	13.7	12.9	11.5	0.38
	Trans	17.3	15.0	15.2	15.6	13.6	12.8	12.0	10.6	0.38
16:1*c*	Control	6.2	6.6	8.0	8.1	7.7	8.7	7.0	6.3	0.44
	Trans	8.0	8.5	6.2	6.3	7.5	8.5	6.2	6.8	0.44
18:0	Control	2.1	1.6	1.2	1.5	1.5	1.1	1.0	1.3	0.11
	Trans	1.9	1.2	1.2	1.3	1.1[b]	1.0	1.1	1.0	0.11
18:1*c*	Control	54.7	55.9	59.1	57.4	58.7	59.0	58.2	62.9	0.83
	Trans	39.7[b]	42.5[b]	44.1[b]	42.2[b]	45.8[b]	45.0[b]	45.2[b]	49.7[b]	0.83
18:1*t*	Control	nd	nd	nd	nd	nd	nd	nd	nd	—
	Trans	9.1	8.6	8.8	8.9	8.5	8.5	9.7	9.4	0.63
18:2n-6	Control	17.8	17.2	16.4	18.1	17.3	16.1	17.4	15.6	0.37
	Trans	18.0	18.6[b]	18.6[b]	18.7	18.4[b]	19.2[b]	19.5[b]	15.1	0.37
20:4n-6	Control	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.3	0.02
	Trans	0.2[b]	0.2[b]	0.3	0.2[b]	0.2[b]	0.3[b]	0.3[b]	0.3	0.02

[a]Values are means of three independent determinations of weight percentage as fatty acid methyl esters, with SEM in the last column; nd, not detected.

[b]Fatty acid value of mice on the Trans Diet was significantly different ($P < 0.05$) from that of mice on the Control Diet in the same age group.

TABLE 4

Fatty Acid Composition of Polar Lipids Isolated from Epididymal Fat Pads of Eight-Month-Old Mice[a]

Fatty acid	Control Diet	Trans Diet	SEM	*P* value
14:0	0.4	0.3	0.02	0.73
16:0	18.7	14.4	2.0	0.24
18:0	17.4	9.6	1.1	0.11
18:1*c*	29.5	25.8	2.3	0.47
18:1*t*	nd	13.1	—	—
18:2n-6	14.1	15.6	1.2	0.51
20:4n-6	7.4	8.7	1.1	0.35
Total (18:1*c* + 18:1*t*)	29.5	35.4		
Total saturated	36.4	24.3		
Total (saturated + 18:1*t*)	36.4	34.0		

[a]Values are means of three independent determinations of the weight percentage as fatty acid methyl ester with the standard error of the mean (SEM) and probability (*P*) values given in the last two columns; nd, not detected.

TABLE 5

Fatty Acid Composition of Nonpolar Lipids Isolated from Epididymal Fat Pads of Eight-Month-Old Mice[a]

Fatty acid	Control Diet	Trans Diet	SEM	*P* value
14:0	0.7	0.9	0.02	0.004
16:0	14.9	14.7	0.2	0.33
18:0	1.5	1.4	0.03	0.59
18:1*c*	57.9	45.2	0.2	
18:1*t*	nd	8.1	0.1	—
18:2n-6	16.3	18.2	0.1	0.005
20:4n-6	0.4	0.2	0.01	0.001
Total (18:1*c* + 18:*t*)	57.9	53.2		
Total saturated	17.1	17.0		
Total (saturated + 18:1*t*)	17.1	25.1		

[a]Values are means of three independent determinations of the weight percentage as fatty acid methyl ester with the standard error of the mean (SEM) and probability (*P*) values given in the last two columns; nd, not detected.

tion may alter cell membrane properties and differentially affect the type and extent of cell damage. In the earlier study (18), the epididymal fat pads were frozen prior to estimating cell size by visual counting techniques, and potentially extensive cell damage with differential effects on membranes of different composition may have occurred.

It was of interest in our study that the diet- and age-related results obtained in the body weight and epididymal fat pad weight (Fig. 1) experiments closely paralleled each other, especially at the older ages. Similar effects of age and diet on body weights of mice have been noted previously (19). Regardless of diet, male mice continued to exhibit body growth till ≈1.5 years of age, after which time the body weights tended to decline sharply. As the average life span for male mice has been described as being 1–2 years (20), the growth curve phenomenon may simply reflect the normal aging process.

Of greater relevance to the present studies were the earlier results obtained with diets containing *t*FA. Compared to other diets, those containing 19 and 61% *t*FA resulted in lower body weights of mice at 13 and 19 mon

of age, but not at the younger ages examined (19). This was similar to our present observations. In the earlier studies (19), differences in body growth due to diet were ascribed to essential fatty acid deficiency, as the 18:2n-6 levels in the diets were different. In our studies, a simple essential fatty acid deficiency was unlikely as the levels of both 18:2n-6 and 18:3n-3 were equivalent in both diets. It is more likely that the presence or absence of *t*FA was responsible for the effect of diet on the growth curve of mice, because the most obvious characteristic common to the earlier (19) and present studies was that mice with lower body weights were chronically fed partially hydrogenated fats.

When the body weights of mice on the Trans Diet were plotted against the percent 18:1*t* in their epididymal fat pads, a statistically significant inverse correlation was obtained (Fig. 2). No such relationship was detected between body weight and other major tissue fatty acids for mice on either diet. Because *t*FA are not synthesized by animal tissues and deposition of 18:1*t* in adipose tissue is highly correlated to the level of dietary 18:1*t* (21), one would expect 18:1*t* in adipose tissue to be a positive marker of food intake. If higher tissue 18:1*t* is a reflection of higher food intake, a positive correlation with body weight would be predicted, rather than the inverse, as was observed herein. In the present studies, mice were fed *ad libitum* and food intake was not measured. However, a role of food intake to explain the above phenomena seems unlikely, as we have data from other studies (22–24) that indicate that food intake between mice fed Trans and Control Diets is quite similar.

Detailed analysis of the fatty acids in epididymal fat tissue (Tables 3–5) revealed several significant patterns. The major adipose tissue saturated fatty acids, 16:0 and 18:0, appeared to decrease with age, while the major *cis*-unsaturated fatty acid, 18:1*c* appeared to increase with age. These findings agree with and further confirm earlier reports (25,26) that show changes in fatty acid composition of adipocytes with age. Comparisons of 18:1*c* in epididymal adipose tissue of mice fed the two diets revealed that the percentages of 18:1*c* in the tissues were not in proportion to the percentages in the diet, suggesting that preferential synthesis or incorporation of 18:1*c* may have occurred in epididymal fat pads of mice fed the Trans Diet.

When the composition of the nonpolar and polar lipids was examined (Tables 4 and 5), the pattern of incorporation of *t*FA resembled that of saturated fatty acids in the polar lipids and that of the *cis*-monounsaturated fatty acids in the nonpolar lipids. Wood (4) observed a similar phenomenon in rats. Although the functional significance of these observations cannot be ascertained from the present studies, it is tempting to suggest that the ability of acylating enzymes to recognize *t*FA differs greatly for different lipid classes.

In the present studies, diet related effects on 14:0, 18:2n-6 and 20:4n-6 were also noted. Levels of both 14:0 and 18:2n-6 tended to be higher in epididymal fat pads of mice fed the Trans Diet, whereas 20:4n-6 tended to be lower. In view of the potential role of 14:0 as an essential component of many acylated proteins (27), the higher levels of myristic acid, which were seen in total and nonpolar lipids, but not in polar lipids, may be important. The effect of *t*FA on 18:2n-6 and 20:4n-6 has been noted previously and has been attributed to inhibition of desaturation in the conversion of 18:2n-6 to 20:4n-6 (28).

Whether the results of these studies can be extrapolated to other species, including humans, is unknown and would require thorough investigation. One of the major problems in extrapolating dietary studies of *t*FA in animals to humans is the lack of information on the *t*FA content of common foods and disagreement regarding the dietary intake of *t*FA in humans (21,29). Recent estimates of the average intake of *t*FA in the United States have been reported to be 8.1 g (29) or 13.3 g (21) per capita. Based on these values and NHANES III (Third National Health and Nutrition Examination Survey) estimates of ≈2,100 calories (30) for the average per capita total food energy intake in the United States, the per capita *t*FA intake would be ≈3.5% or ≈5.7% of total calories in humans. In our mouse study, the 18:1*t* represented 5.6% of the total calories, and the minimum required to observe effects on adipose tissue was not determined.

Although the threshold required for the effect is unknown, it is clear that chronic consumption of diets containing partially hydrogenated fat influences lipid metabolism and the amount of lipid deposited in adipose tissue of mice. The results are consistent with an effect of dietary *t*FA on the synthesis or breakdown of triacylglycerols; however, the underlying mechanisms involved are unclear. Various metabolic factors, including lipogenic enzymes, influence fat storage. Additional studies are underway to examine the effects of dietary *t*FA on selected lipogenic enzyme activities in adipose cells.

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Liver Δ5 and Δ6 Desaturase Activity Differs Among Laboratory Rat Strains

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This study was designed to examine the variations among rat strains in hepatic fatty acid desaturase activities and to determine the correlations between the activities of these enzymes and the levels of each microsomal fatty acid. Wistar rats from two different sources as well as Long-Evans and Sprague-Dawley rats were selected to assess, under standard and identical experimental conditions, the liver Δ5 and Δ6 desaturase activities. Both desaturase activities were significantly reduced by 56% in Sprague-Dawley rats when compared to BB-Wistar control rats, whereas intermediate reduced values were detected in Wistar (CR) and Long-Evans strains. The activities of Δ5 and Δ6 desaturases were significantly and positively correlated with each other. However, no significant correlations were detected between either Δ5 or Δ6 desaturase activities and levels of any of their fatty acid substrates or any other of the major microsomal fatty acids. Fatty acid composition of microsomal total lipids showed strain dependency. A positive correlation was detected between the microsomal levels of the two major final products of both desaturases, namely 20:4n-6 and 22:6n-3. In general, the sum of n-3 or n-6 fatty acids but not the ratio of one to the other, varied among rat strains. The study demonstrated that Δ6 and Δ5 desaturase activities are strain-related. The data also suggested that (i) the desaturation activity should be measured and not predicted from the fatty acid composition and (ii) different rat strains should be used for lipid metabolic studies before conclusions are drawn for rats in general.
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There are several fatty acid desaturases that are membrane-bound and immunologically distinct enzymes (1,2). Delta-5 and Δ6 desaturases are required to convert dietary linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) to their metabolites, such as dihomo-γ-linolenic acid (20:3n-6), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), for membrane phospholipid and eicosanoid synthesis (3). Polyunsaturated fatty acids of the n-9 type are also produced by these enzymes. Age, season (4), circadian rhythm (5), disease, hormonal (6) and dietary status (7) are factors that alter Δ5 and Δ6 desaturase activities through different mechanisms (8). The activities of these enzymes also vary between animal species as well as between different organs of the same species (9,10). For example, there is a lack or near lack of Δ6 desaturase in felines (11) while rat hepatocytes desaturate linoleic acid rapidly relative to the rates found in rat heart or kidneys (10).

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; FAME, fatty acid methyl ester; HPLC, high-performance liquid chromatography.

Rat liver microsomes are the most thoroughly studied system and are generally considered as the standard model for desaturation experiments (12). However, the rat strains used in the studies differ from one laboratory to another, and in some cases, in the same laboratory between experiments.

Rats of different genetic origin are known to differ in various biological and biochemical responses, including their susceptibility to adjuvant disease (13), their chylomicron metabolism and cholesterolemic response (14), and in their resistance to the myocardial lesions caused by dietary erucic acid (15). Dietary fatty acids also affect plasma lipids and 5-lipoxygenase products of different rat strains in different ways (16,17). However, the variations in the activity of fatty acid desaturases among rat strains had not been examined previously.

We therefore determined the liver Δ5 and Δ6 desaturase activities of four commonly used strains of laboratory rats under identical experimental conditions. The correlations between the two enzyme activities and between the microsomal fatty acid concentrations are also presented.

MATERIALS AND METHODS

Chemicals and radiochemicals. All organic solvents and chemicals were of reagent grade and were obtained from British Drug House Inc. (Toronto, Ontario, Canada). Lipid standards were from Nu-Chek-Prep (Elysian, MN). Niacinamide, *N*-acetylcysteine, ATP, co-enzyme A and NADH were obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Linoleic and [1-^{14}C]dihomo-γ-linolenic acids (99% radiochemical purity; 53.0 and 54.9 mCi/mmol, respectively) were purchased from DuPont Canada Inc. (Markham, Ontario, Canada).

Animals and diets. Female Wistar (CR), Long-Evans and Sprague-Dawley rats were obtained from Charles River Canada Inc. (St. Constant, Quebec, Canada). Female BB-Wistar control rats were kindly provided by the Health Protection Branch of Health and Welfare (Ottawa, Ontario, Canada). Animals were housed three per cage in a temperature-controlled environment (22 ± 2°C) with a 12 h light/dark cycle and were fed Rodent Laboratory Chow No. 5001 (Ralston Purina Co., St. Louis, MO) and water, *ad libitum*, for 7 d immediately after arrival. All rats were two months old weighing 190 ± 5 g at the time of sacrifice.

Isolation of hepatic microsomes. Nonfasted rats were sacrificed by cervical dislocation at 9:30 a.m. to avoid variations in enzyme activity caused by circadian rhythm (5) and to obtain a substantial and even, though not maximal, activity of liver desaturase (5,7). Livers were quickly rinsed with cold 0.9% NaCl solution, weighed and minced with scissors. All procedures were performed at 4°C unless specified otherwise. Micro-

somes were isolated by differential ultracentrifugation as previously described (18). Briefly, livers were homogenized in a solution (1:3, wt/vol) containing 0.25 M sucrose, 62 mM potassium phosphate buffer (pH 7.0), 0.15 M KCl, 1.5 mM *N*-acetylcysteine, 5 mM $MgCl_2$ and 0.1 mM ethylenediaminetetraacetic acid (EDTA) using four strokes of a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 10,400 × *g* for 20 min to eliminate mitochondria and cellular debris. The supernatant was filtered through a three-layer cheesecloth and was centrifuged at 105,000 × *g* for 60 min. The microsomal pellet was gently resuspended in the same homogenization solution with a small glass/Teflon homogenizer and kept frozen at −70°C until used, as reported elsewhere (19). The absence of mitochondrial contamination was enzymatically assessed as previously described (20). The protein concentration was measured by the method of Lowry *et al.* (21) with bovine serum albumin as the standard.

Desaturase assays. The activities of Δ5 and Δ6 desaturases were determined by measuring the conversion of [1-^{14}C]20:3n-6 (dihomo-γ-linolenic acid) to [1-^{14}C]20:4n-6 (arachidonic acid) and of [1-^{14}C]18:2n-6 (linoleic acid) to [1-^{14}C]18:3n-6 (γ-linolenic acid), respectively. The conditions for the desaturation assays were those described by Leikin and Brenner (22), under which the enzymes are saturated with substrates and the reactions are linear with incubation time. Reactions were started by adding 2 and 3 mg of microsomal protein for Δ5 and Δ6 desaturase, respectively, to preincubated tubes containing 0.20 μCi of the substrate fatty acid at a final concentration of 33.3 μM in 1.5 mL of the homogenization solution, containing NaF (42 mM), niacinamide (0.33 mM), ATP (1.57 mM), NADH (1.01 mM) and coenzyme A (0.09 mM) as described (22). The tubes were vortexed vigorously, and after 15 min incubation in a shaking water bath (37°C) the reactions were stopped by the addition of 2 mL of 10% (wt/vol) KOH in ethanol. Lipids in the incubation mixture were saponified at 80°C for 45 min under N_2. The samples were then left in ice for 5 min before acidification. The fatty acids were extracted with hexane and esterified with BF_3/methanol at 90°C for 30 min (23).

Radiolabeled fatty acid methyl esters (FAME) were analyzed as previously described (24). Analyses were done by high-performance liquid chromatography (HPLC) using a Waters instrument (Waters Associates, Milford, MA) equipped with a variable wavelength ultraviolet-vis monitor (set at 205 nm), a radioisotope detector (model 171; Beckman, Palo Alto, CA) with a solid scintillator cartridge (97% efficiency for ^{14}C-detection) and an ultrasphere ODS column 25 cm × 4.6 mm i.d. (5 μm particle size; Beckman). FAME were separated isocratically with acetonitrile/water (95:5, vol/vol) at a flow rate of 1 mL/min, and were identified by comparison with authentic standards.

Lipid analysis. Lipids were extracted from liver microsomes with chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (25) and were transesterified to form methyl esters as previously described (23). The fatty acid composition of microsomal total lipids was determined by gas–liquid chromatography using a Hewlett-Packard instrument (model 5880; Avondale, PA) equipped with flame-ionization detector and a glass column (2 mm i.d. × 180 mm) packed with GP 10% SP-2330 (Supelco Canada Ltd., Oakville, Ontario, Canada) as previously described (26).

Statistical analysis. The results are expressed as means ± standard deviation. The significance of differences was determined using a two-tailed Student's *t*-test. A difference was considered significant at $P < 0.01$ unless specified otherwise in the text. Regression analysis was by the least-squares method.

RESULTS

Strain variations in hepatic Δ5 and Δ6 desaturase activity are shown in Figure 1. Both desaturase activities were significantly reduced by 56% in Sprague-Dawley rats when compared to BB-Wistar control rats, whereas intermediate reduced values were detected in Wistar (CR) and Long-Evans strains. A linear regression analysis of these data showed a significant positive correlation (correlation coefficient, r = 0.81; $P < 0.01$) between Δ5 and Δ6 desaturase activities (Fig. 2). This figure shows two areas defined by the desaturase activities of Sprague-Dawley and BB-Wistar control rats that enclose a third area determined by the Long-Evans and Wistar-CR desaturase activities.

The fatty acid composition of the microsomal total lipids showed strain dependency (Table 1). The percentages of the major polyunsaturated fatty acids, 20:4n-6 and docosahexaenoic acid (22:6n-3), and the sum of n-6 fatty acids minus 18:2n-6 were significantly lower in Sprague-Dawley rats as compared to the other three strains. This decrease was balanced by an increase in palmitic (16:0), linoleic (18:2n-6) and eicosapentaenoic

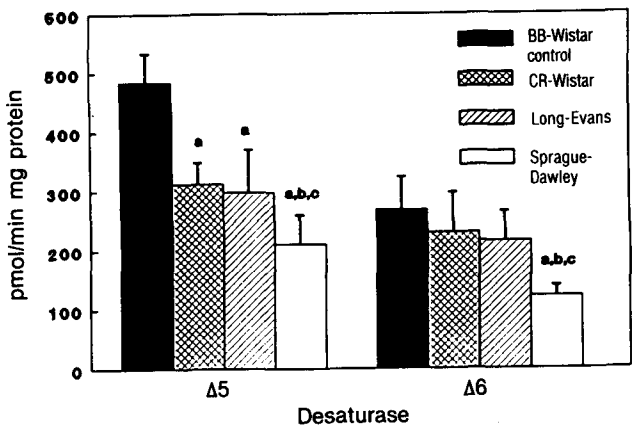

FIG. 1. Liver microsomal Δ5 and Δ6 desaturase activity in four rat strains. Animals were treated and incubations were performed under identical experimental conditions. The activities of Δ5 and Δ6 desaturases were determined by measuring the conversion of [1-^{14}C]20:3n-6 to [1-^{14}C]20:4n-6 and of [1-^{14}C]18:2n-6 to [1-^{14}C]18:3n-6, respectively, as detailed under the Materials and Methods section. Values are the means ± SD of at least four animals. The superscripts denote statistically significant differences ($P < 0.01$) to [a]BB-Wistar control, [b]Wistar-CR and [c]Long-Evans.

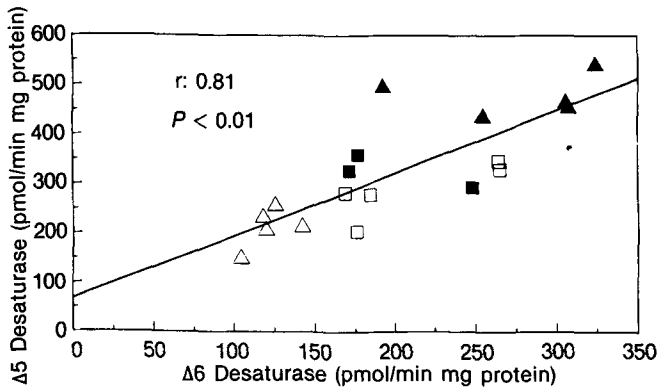

FIG. 2. Relationship between Δ5 and Δ6 desaturase activities of (▲) BB-Wistar control, (■) Wistar-CR, (□) Long-Evans and (△) Sprague-Dawley rats. The activities of Δ5 and Δ6 desaturases were determined by measuring the conversion of [1-^{14}C]20:3n-6 to [1-^{14}C]20:4n-6 and of [1-^{14}C]18:2n-6 to [1-^{14}C]18:3n-6, respectively, as detailed under the Materials and Methods section. Regression analysis was by the least-squares method. Each point represents the mean of duplicate determinations.

(20:5n-3) acid concentrations. In general, the sum of n-3 and n-6 fatty acids, but not the ratio of the two, varied significantly among the strains. Table 1 also shows that the ratios of Δ5 and Δ6 desaturase activities were not significantly different among the rat strains studied.

Linear regression analyses were performed relating the concentration of 17 major microsomal fatty acids to the Δ5 or Δ6 desaturase activity. The 18:2n-6 levels were modestly and negatively correlated with Δ5 desaturase activity (r:0.51, $P < 0.05$); no significant correlations were found with any fatty acid concentration and Δ6 desaturase activity.

The relationships between the microsomal levels of various pairs of fatty acids were also assessed. In general, no significant correlations were seen except for the following: Stearic acid (18:0) and 16:0 contents were negatively correlated with 20:4n-6 and 22:6n-3 levels, respectively (Figs. 3 and 4). A positive correlation was seen between 20:4n-6 and 22:6n-3 contents (Fig. 4C). Eicosapentaenoic acid (20:5n-3) levels were positively correlated with the content of its precursor, α-linolenic acid (18:3n-3) (Fig. 5B) and with the levels of its elongation product, docosapentaenoic acid (22:5n-3) ($r = 0.58$, $P < 0.02$, data not shown). Linoleic acid (18:2n-6) content also correlated positively with 20:5n-3 levels (Fig. 5A).

DISCUSSION

Wistar rats from two different sources and two other commonly used laboratory rat strains were selected to assess liver Δ5 and Δ6 desaturase activities and to determine the relationship between the activities of these enzymes and the levels of individual microsomal fatty acids. All strains were examined under precisely identical conditions to facilitate comparisons.

The data show that the desaturase activities are clearly strain related (Fig. 1). Since both Δ5 and Δ6 desaturase activities were significantly and positively correlated (Fig. 2) and since there was an approximately constant ratio between these activities (Table 1), a proportional flow of n-3 and n-6 polyunsaturated fatty acids

TABLE 1

Fatty Acid Composition (%) of Liver Microsomes from Different Rat Strains[a]

Fatty acid	BB-Wistar control	CR-Wistar	Long-Evans	Sprague-Dawley
16:0	12.4 ± 0.8	11.7 ± 0.7	11.8 ± 0.5[d]	13.4 ± 0.6[c,d]
16:1	1.0 ± 0.1	0.5 ± 0.1[c]	0.5 ± 0.1[c]	0.7 ± 0.3
18:0	20.7 ± 1.4	19.7 ± 1.3	17.9 ± 1.7[c]	24.1 ± 5.2[e]
18:1	7.2 ± 0.5	6.1 ± 0.6[c]	7.4 ± 0.5[d]	7.4 ± 0.7[d]
18:2n-6	12.6 ± 1.4	13.5 ± 1.6	14.2 ± 0.9[c]	14.9 ± 1.6[c]
18:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
18:3n-3	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:3n-6	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.2
20:4n-6	30.0 ± 0.9	31.6 ± 0.8[c]	30.7 ± 1.2	24.4 ± 3,4[c,d,e]
20:5n-3	1.2 ± 0.2	1.6 ± 0.4	1.9 ± 0.5[c]	1.9 ± 0.5[c]
22:5n-3	0.9 ± 0.1	1.1 ± 0.1[c]	1.2 ± 0.1[c]	1.0 ± 0.2
22:6n-3	10.4 ± 1.0	11.5 ± 1.1	10.8 ± 0.5	8.4 ± 0.8[c,d,e]
Total (n-6)—LA	32.0 ± 0.8	33.3 ± 0.9	32.5 ± 1.0	26.4 ± 3.2[c,d,e]
Total (n-6)	44.6 ± 1.6	46.8 ± 1.0[c]	46.7 ± 0.8[c]	41.3 ± 4.8[d]
Total (n-3)	12.7 ± 1.1	14.5 ± 0.8[c]	14.1 ± 1.0[c]	11.5 ± 1.2[d,e]
Total MUFA	8.1 ± 0.5	6.5 ± 0.6[c]	7.9 ± 0.6[d]	8.1 ± 0.6[d]
Total SFA	33.4 ± 1.1	31.5 ± 1.0[c]	29.9 ± 1.5[c]	37.7 ± 5.3[d,e]
n-6/n-3	3.6 ± 0.4	3.2 ± 0.2	3.3 ± 0.3	3.6 ± 0.3
Δ5/Δ6[b]	1.9 ± 0.6	1.7 ± 0.4	1.4 ± 0.2	1.8 ± 0.2

[a]Abbreviations: LA, linoleic acid, 18:2n-6; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; Δ5/Δ6, ratio of Δ5 to Δ6 desaturase activities. Minor fatty acids make up the balance. Values are the mean of three rats ± SD.

[b]Data from Figure 1.

[c,d,e]Significantly different ($P < 0.01$, two-tailed Student's t-test) to BB-Wistar-control, CR-Wistar and Long-Evans, respectively.

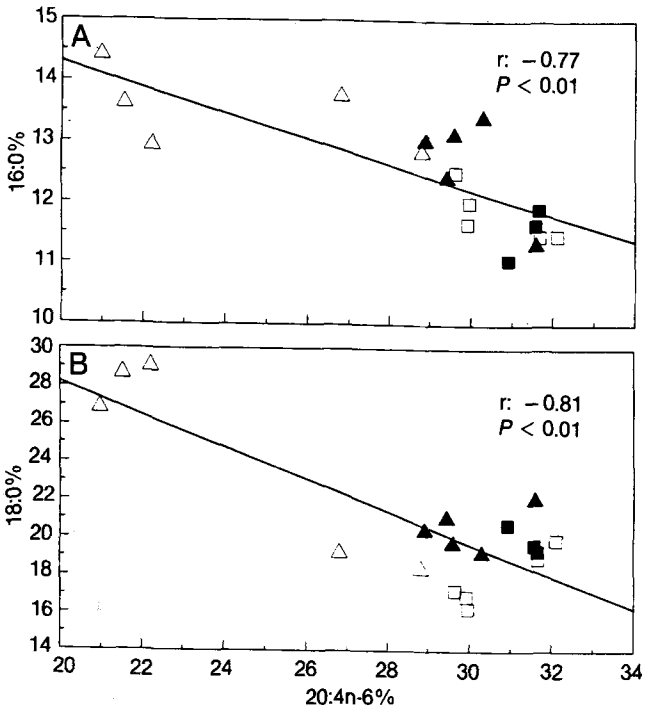

FIG. 3. Relationships between the 20:4n-6 and 16:0 or 18:0 percentages of total lipid microsomal fatty acid composition of (▲) BB-Wistar control, (■) Wistar-CR, (□) Long-Evans, and (△) Sprague-Dawley rats. Lipids were extracted from liver microsomes with chloroform/methanol. The fatty acid composition of microsomal total lipids was determined by gas–liquid chromatography. Regression analysis was by the least-squares method. Each point represents the mean of duplicate determinations.

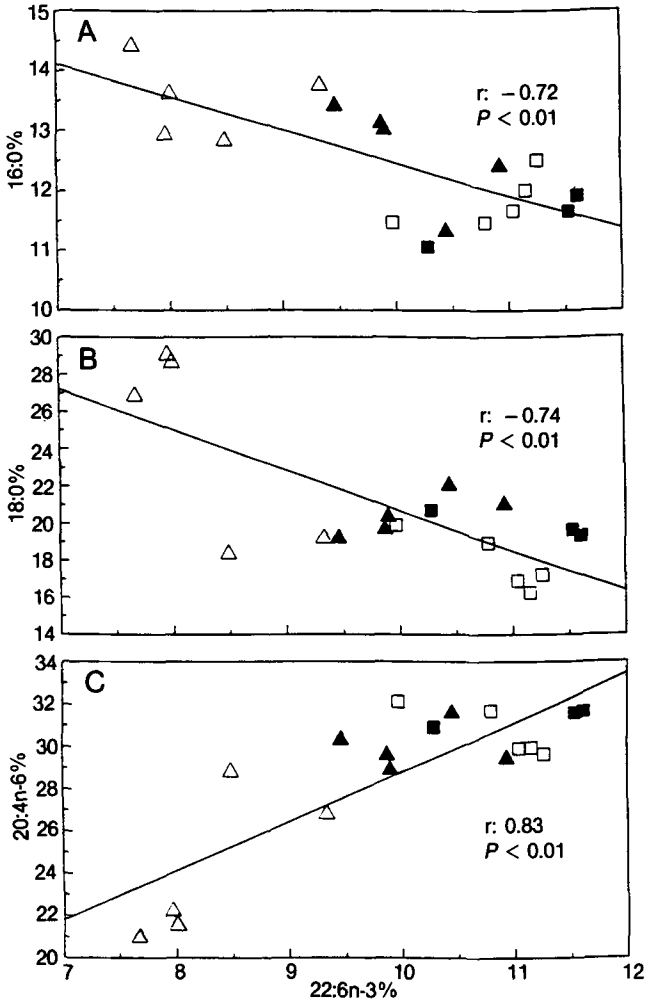

FIG. 4. Relationships between the 22:6n-3 and 16:0, 18:0 or 20:4n-6 percentages of total lipid microsomal fatty acid composition of (▲) BB-Wistar control, (■) Wistar-CR, (□) Long-Evans and (△) Sprague-Dawley. Lipids were extracted from liver microsomes with chloroform/methanol. The fatty acid composition of microsomal total lipids was determined by gas–liquid chromatography. Regression analysis was by the least-squares method. Each point represents the mean of duplicate determinations.

without any accumulation or excessive synthesis of post-Δ6 and/or post-Δ5 desaturation products seems to be assured in all strains. This also explains the positive correlation found between the microsomal levels of the two major final products formed by the two desaturases, namely 20:4n-6 and 22:6n-3 (Fig. 4C), and in the relatively constant ratio of the sum of n-6 to the sum of n-3 fatty acids (Table 1). On the other hand, the high desaturase activities found in certain strains are not mirrored in high levels of 20:5n-3 (post-Δ6 and post-Δ5 desaturation products) or in a low content of the precursor, α-linolenic acid (18:3n-3) (Figs. 2 and 5). It is noteworthy that no significant correlations were found between either Δ6 or Δ5 desaturase activities and any of their fatty acid substrates or products or any other of the major microsomal fatty acids. Our data expand on those previously reported on Wistar rats and support the concept that desaturase activities are not a major controlling factor in establishing liver microsomal fatty acid composition (27). Thus desaturation activity should be measured and should not be predicted from the fatty acid composition. We have previously reported that the total fatty acid composition of liver did not significantly differ from that of its microsomes (28). Thus, it is probably that the correlations, or the lack of them, with regard to microsomal fatty acid levels are also representative of the whole liver. Increased levels of 20:4n-6 and 22:6n-3 were counterbalanced by a decrease in the content of saturated fatty acids (16:0 and 18:0), which in turn serve as substrates of the Δ9 desaturase. Brenner (10) could not detect relationships between Δ6 and Δ9 desaturase activities when both enzymes were measured simultaneously in the same microsomes of rats with different hormonal or dietary treatments. The analyses of all data taken together lead us to suggest that polyunsaturated fatty acid synthesis is coordinated and that monoenoic fatty acids are not involved in modifying the proportional Δ6 desaturation of fatty acids of the n-6 and n-3 families.

Since the desaturase activities and the microsomal fatty acid composition are different in each rat strain, the lipid metabolic response to different stimuli (disease, hormones, dietary treatment, etc.) may also be

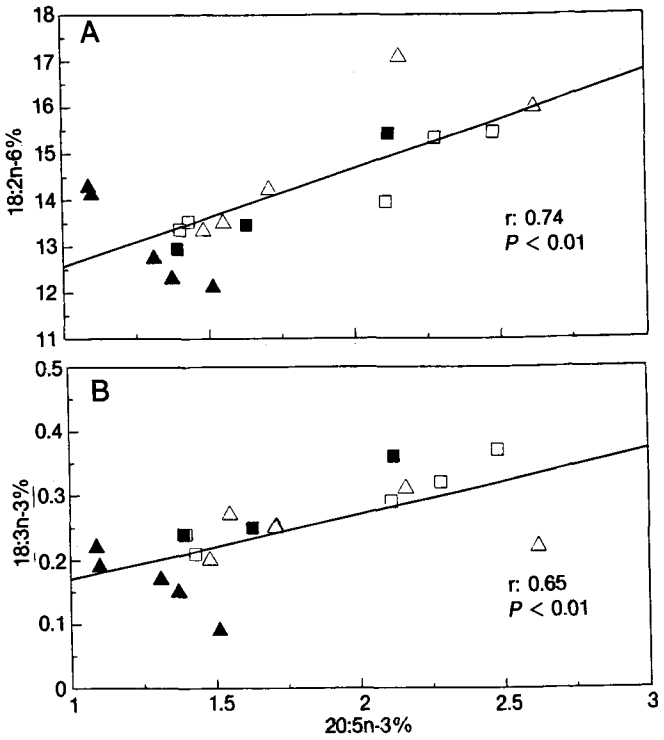

FIG. 5. Relationships between the 20:5n-3 and 18:2n-6 or 18:3n-3 percentages of total lipid microsomal fatty acid composition of (▲) BB-Wistar control, (■) Wistar-CR, (□) Long-Evans and (△) Sprague-Dawley. Lipids were extracted from liver microsomes with chloroform/methanol. The fatty acid composition of microsomal total lipids was determined by gas–liquid chromatography. Regression analysis was by the least-squares method. Each point represents the mean of duplicate determinations.

strain-dependent. We propose that several rat strains should be used for lipid metabolism studies before conclusions are drawn for the rat species as a whole. Extrapolations from one species to another are likely to be unreliable.

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Lipoprotein Lipase and Hepatic Lipase in Wistar and Sprague-Dawley Rat Tissues. Differences in the Effects of Gender and Fasting

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To evaluate the effects of strain, gender and fasting in the regulation of lipoprotein lipase (LPL) and hepatic lipase (HL) activities were measured in tissues of male and female Wistar and Sprague-Dawley rats after feeding or a 24-h starvation period. It is noteworthy that an effect of gender on LPL activity was observed in Wistar, but not in Sprague-Dawley rats, not only in the basal (fed) activity in several tissues, such as white and brown adipose tissues, heart, and brain, but also in response to fasting which affected LPL activity in brown adipose tissue, heat and lung of female but not of male Wistar rats. By contrast, HL activity in liver, plasma and adrenals of Sprague-Dawley rats was higher in females than in males. No effect of gender on HL activity was observed in Wistar rats. Our results indicate that differences exist between Wistar and Sprague-Dawley rats in the regulation of both LPL and HL. Some of the contradictory results found in the literature may be explained by the differences between rat strains and gender, as well as differences in the nutritional status of the animals.

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Lipoprotein lipase (E.C. 3.1.1.34; LPL) and hepatic lipase (HL) are lipolytic enzymes that are involved in the metabolism of circulating lipoproteins. LPL catalyzes the hydrolysis of circulating triacylglycerols in chylomicra and in very low density lipoproteins (1). Many tissues express the LPL gene, but LPL activity is particularly high in adipose tissue, heart and skeletal muscles, and in lactating mammary gland (2). The liver also expresses high levels of LPL activity during the perinatal period (3). The enzyme is functional at the luminal side of the endothelial cells, but is synthesized inside the parenchymal cells (4–6). HL participates in the clearance of cylomicron remnants by the liver (7) and in the conversion of high density lipoproteins (HDL_2 into HDL_3) thus contributing to the reverse cholesterol transport to the liver (see Ref. 8 for review). It is important that HL is also located at the vasculature (9), although the enzyme is synthesized within the hepatocytes (10). The presence of a lipolytic activity with characteristics similar to those of hepatic lipase was also demonstrated in some steroidogenic tissues, such as adrenals and ovaries in several animal species (11,12), although the enzyme does not appear to be synthesized in these tissues (13,14). It was therefore proposed that the hepatic lipase found in these tissues may originate in the liver (13). Low but significant levels of HL activity were also reported in the plasma of rats (15), as well as of humans (16).

Both LPL and HL are under nutritional control. LPL is increased in white adipose tissue (WAT) but is decreased in heart of fasted rats (see Refs. 17 and 18 for review), although some of the data reported in the literature are in conflict (4,19–22). We have recently observed that the effect of fasting on LPL activity in the liver of neonatal rats depends on the rat strain used as model (23). We have therefore studied the differences between strains in the effect of fasting on both LPL and HL activities in various tissues.

MATERIALS AND METHODS

Animals. Wistar and Sprague-Dawley rats were obtained from Charles River (Barcelona, Spain). Animals were sacrificed by decapitation at the age of 60 d, and tissues [liver, heart, lungs, epididymal or periuterine WAT, interscapular brown adipose tissue (BAT), adrenal glands and brain] were immediately excised, cleaned and frozen in liquid N_2. The blood was collected in heparinized vials, and plasma was obtained by centrifugation (30 min at 1,000 × *g* at 4°C). Plasma was kept at –40°C until used to determine lipolytic activities. Tissue homogenates were prepared in 10 mM Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], pH 7.5, containing 1 mM ethylenediaminetetraacetate and 1 mM dithiothreitol using a Polytron (Kinematica GmbH, Luzern, Switzerland) homogenizer. Homogenates were cleared up by centrifugation (10 min at 10,000 × *g* at 4°C). When necessary, the upper fatty layer was removed, and the supernatant centrifuged again until no lipid layer remained.

LPL assay. LPL activity was determined as described (24). The assay mixture contained 0.6 mM glycerol tri[9,10(n)-^{3}H]oleate (12 Ci/mol), 50 mM $MgCl_2$, 0.05% albumin (fatty acid-free), 3% serum (preheated for 60 min at 50°C), 25 mM Pipes [piperazine-*N,N'-bis*(2-ethanesulfonic acid)], pH 7.5, and 0.02 mL of sample in a final volume of 0.2 mL. The incubation was carried out for 30 min at 25°C. The reaction was terminated, and the released [^{3}H]oleate was quantified, as previously described (25). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of oleate per min.

HL assay. HL activity was determined as previously described (26). The assay mixture contained 2.5 mM glycerol tri[9,10(n)-^{3}H]oleate (0.3 Ci/mol), 0.75 M NaCl, 3% albumin (fatty acid-free), 50 mM Tris [tris(hydroxymethyl)-aminomethane], pH 8.5, and 0.05 mL of sample in a final volume of 0.2 mL. The incubation was carried out for 30 min at 25°C. The reaction was terminated, and the released [^{3}H]oleate was quantified as described above for LPL.

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Abbreviations: BAT, brown adipose tissue; HDL, high density lipoproteins; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HL, hepatic lipase; LPL, lipoprotein lipase; Pipes, piperazine-*N,N'-bis*(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; WAT, white adipose tissue.

RESULTS

Effect of gender. At the age of 60 d, the body weight was lower in Wistar than in Sprague-Dawley rats (Table 1). Body weights of females were about 40% lower than those of males in both strains. Similar differences were also observed in several tissues, notably in liver, weights, but also in heart and lung weights.

In both Wistar and Sprague-Dawley rats, the distribution of LPL activity between tissues was similar (Table 2). However, gender affected LPL activity in tissues of Wistar but not in tissues of Sprague-Dawley rats. Thus, in Wistar rats LPL activity in WAT and brain was lower in females than in males, but the activity in heart and BAT was higher in females than in males. LPL activity was not detectable in liver and plasma of adult animals (data not shown). The lack of an effect of gender on LPL activity in Sprague-Dawley rats has already been reported (27).

We studied HL activity in liver, plasma and adrenals (Table 3). As previously described (11,12), we found that HL activity was higher in liver than in adrenals and that a substantial amount could be detected in plasma. No effect of gender was seen in Wistar rats (this agrees with previous results from our laboratory; see Ref. 28), but in Sprague-Dawley rats HL activity was 35–55% higher in females than in males, not only in the liver but also in plasma and adrenals.

As shown in Table 3, fasting caused a decrease in the HL activity not only in liver but also in plasma of Wistar and Sprague-Dawley rats. No significant differences were found between female and male animals in their response to fasting. In adrenals, fasting also produced a decrease in HL activity, but the differences were only significant in male Wistar and in female Sprague-Dawley rats.

DISCUSSION

Several studies have demonstrated differences in both adipose tissue (29–31) and postheparin plasma (32) LPL activity between men and women. For rodents, scarce and conflicting results have been reported (27,33,34). Our data indicate that the effect of gender on both tissue LPL and HL very much depends on the rat strain used.

The effect of gender on LPL activity is likely to be related to the role of estrogens. It is well established that, in female rats, ovariectomy increases LPL activity in WAT (33), and estrogen treatment restores the lower LPL activity in this tissue (33,5). In the heart, LPL activity is increased by estrogen treatment (27). Androgens may not be directly involved in the effect of gender on LPL, as castration of male rats did not produce any alteration in adipose tissue LPL activity (27). In humans, where LPL activity in WAT is higher in women than in men, estrogen treatment increases LPL activity (see Ref. 17 for review). The role of estrogen in the effect of gender on HL is not clear for the rat. Malendowicz and Paluszak (36) found that ovariectomy had no effect on HL-like activity in adrenals, and Staels *et al.* (37) described that the increase in both hepatic lipase activity and *m*RNA relative content in the liver of ovariectomized rats was not observed in pair-fed animals. They also found no differences between female and male Wistar rats in HL *m*RNA relative content in the liver (37). Androgens are more likely involved in the effect of gender on hepatic lipase, as it was reported that orchiectomy resulted in an increase in the HL-like activity in adrenals, and that testosterone-replacement therapy restored normal values (36). In humans, the relationship between estrogens and hepatic lipase is well established (38). Estrogen administration to postmenopausal women decreased postheparin plasma HL activity (39), even in diet-controlled trials (40). Furthermore, changes in postheparin plasma HL activity were countercurrent to those of 15-β-estradiol in the course of the estral cycle (41). In addition, HL activity in postheparin plasma was shown to be higher in men than in women (42). Therefore, profound differences between rats and humans appear to exist in the effect of sex hormones on both LPL and HL. Our results further suggest that differences between rat strains may exist in the effects of these hormones.

TABLE 1

Body and Tissue Weight of Wistar and Sprague-Dawley Rats[a]

	Wistar			Sprague-Dawley		
	Male	Female	*P* vs. male	Male	Female	*P* vs. male
Body weight (g)	318 ± 4	188 ± 1	<0.001	370 ± 4	238 ± 1	<0.001
Tissue weight (g)						
WAT	1.85 ± 0.17	2.06 ± 0.10	NS	3.22 ± 0.41	2.89 ± 0.14	NS
BAT	0.18 ± 0.02	0.14 ± 0.03	NS	0.39 ± 0.02	0.29 ± 0.03	NS
Heart	0.85 ± 0.01	0.59 ± 0.02	<0.001	0.99 ± 0.03	0.76 ± 0.01	<0.01
Lung	1.40 ± 0.04	1.01 ± 0.01	<0.001	1.37 ± 0.03	1.17 ± 0.03	<0.01
Brain	1.38 ± 0.03	1.31 ± 0.03	NS	1.41 ± 0.02	1.33 ± 0.04	NS
Liver	13.96 ± 0.09	7.78 ± 0.44	<0.001	15.59 ± 0.42	9.55 ± 0.19	<0.001
Adrenals	0.058 ± 0.008	0.068 ± 0.004	NS	0.062 ± 0.003	0.064 ± 0.007	NS

[a]Results are the means ± SE of 5–6 animals per group. Statistical comparisons were made by Student's *t*-test. NS, nonsignificant differences; WAT, white adipose tissue (gonadal); BAT, brown adipose tissue (interscapular).

TABLE 2

Lipoprotein Lipase Activity in Tissues of Wistar and Sprague-Dawley Rats[a]

	Wistar			Sprague-Dawley		
	Fed	Fasted	*P* vs. fed	Fed	Fasted	*P* vs. fed
White adipose tissue						
Male	78 ± 5	15 ± 5	<0.001	69 ± 10	14 ± 1	<0.001
Female	53 ± 5	11 ± 1	<0.001	69 ± 11	15 ± 1	<0.001
P vs. male	<0.05			NS		
Brown adipose tissue						
Male	42 ± 7	31 ± 6	NS	30 ± 3	26 ± 3	NS
Female	68 ± 5	19 ± 4	<0.001	27 ± 8	26 ± 8	NS
P vs. male	<0.05			NS		
Heart						
Male	137 ± 9	140 ± 11	NS	129 ± 8	334 ± 34	<0.01
Female	162 ± 6	189 ± 5	<0.05	130 ± 14	200 ± 13	<0.01
P vs. male	<0.05			NS		
Lung						
Male	44 ± 8	59 ± 10	NS	55 ± 4	63 ± 6	NS
Female	51 ± 4	69 ± 2	<0.01	66 ± 4	63 ± 12	NS
P vs. male	NS			NS		
Brain						
Male	4.3 ± 0.6	3.6 ± 0.4	NS	3.3 ± 0.3	2.5 ± 0.2	NS
Female	2.9 ± 0.1	3.1 ± 0.3	NS	3.1 ± 0.2	2.4 ± 0.3	NS
P vs. male	<0.05			NS		

[a]Tissues were obtained from 60-day-old rats either fed or fasted and processed to determine lipoprotein lipase activity. Results are expressed in mU/g tissue and are means ± SE of 5–6 animals per group. Statistical comparisons were made by Student's *t*-test. NS, nonsignificant differences.

There is a general agreement in the literature concerning the effect of fasting on PL activity in WAT (see Ref. 17 for review), and we have found that LPL activity in this tissue was decreased by fasting in all animals studied. For the heart, there exists some consensus that fasting increases LPL activity, but some studies failed to show such an effect (see Ref. 18 for review). Our results indicate that this apparent contradiction may be due, at least in part, to the gender or the strain of rats used in these studies, as we found that fasting produced a stronger effect in the heart of Sprague-Dawley rats (about a 2-fold increase) than in the heart of Wistar rats (1.2-fold increase in females and no effect in males).

LPL activity in rat brain is known to be decreased by extended (three days) fasting (43,44). Our results, indicating no effect of 24-h fasting, are in agreement with those reported by Gavin *et al.* (44). Little is known about the effect of the nutritional status on LPL activity in BAT and lung. Very early studies by Hamosh and Hamosh (45) on the effect of fasting in Sprague-Dawley rats showed no effect on

TABLE 3

Hepatic Lipase Activity in Tissues of Wistar and Sprague-Dawley Rats[a]

	Wistar			Sprague-Dawley		
	Fed	Fasted	*P* vs. fed	Fed	Fasted	*P* vs. fed
Liver (mU/g)						
Male	633 ± 33	433 ± 21	<0.001	546 ± 18	372 ± 22	<0.001
Female	627 ± 31	494 ± 36	<0.05	739 ± 29	473 ± 32	<0.001
P vs. male	NS			<0.05		
Plasma (mU/mL)						
Male	6.6 ± 0.4	3.9 ± 0.2	<0.001	5.9 ± 0.4	3.9 ± 0.2	<0.01
Female	6.2 ± 0.2	4.9 ± 0.5	<0.05	8.1 ± 0.5	4.7 ± 0.4	<0.001
P vs. male	NS			<0.05		
Adrenals (mU/g)						
Male	119 ± 5	77 ± 5	<0.01	233 ± 18	196 ± 13	NS
Female	97 ± 11	79 ± 5	NS	360 ± 37	209 ± 50	<0.05
P vs. male	NS			<0.05		

[a]Hepatic lipase activity was determined in the liver, plasma and adrenals of Wistar and Sprague-Dawley rats, fed or fasted (24 h). Results are means ± SE of five animals per group. Statistical differences were determined by Student's *t*-test. NS, nonsignificant differences.

LPL activity in lungs. In BAT of male Wistar rats, it was shown that after three days of fasting, LPL activity in this tissue was decreased (46). Our results showing that fasting affected LPL activity in both BAT and lung only in female Wistar rats are consistent with these reports.

The results indicate that in Wistar rats differences exist between females and males not only in the LPL activity in several tissues, but also in their response to fasting. This suggests that sex hormones may affect the action of hormones directly responsible for the effect of fasting. Differences between female and male rats in their response to hormones have been described. For example, the sensitivity of hepatocytes to the stimulation of glycogen phosphorylase by adrenaline is an order of magnitude higher in male than in female rats (47). This is known to be due to the different mechanism of action of catecholamines in the liver, i.e., the α_1-receptor-mediated calcium mobilization in males and the β-receptor-mediated cAMP increase in females (48). Also, the sensitivity of adipocytes to stimulation of glucose metabolism by insulin is greater in female than in male rats (49). Furthermore, while in hepatocytes from male rats prostaglandins of the E series inhibit hormone-stimulated glycogenolysis, in hepatocytes from female rats they do not (50). The mechanisms by which sex hormones produce these differences are largely unknown. The relationship between sex and other hormones in the regulation of LPL in rat tissues has not yet been explored. To address this question and to elucidate the reasons for the differences in the response of LPL to fasting between rat strains will give new insights into how this important enzyme of lipoprotein metabolism is regulated.

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Diets Rich in Lean Beef Increase Arachidonic Acid and Long-Chain ω3 Polyunsaturated Fatty Acid Levels in Plasma Phospholipids

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Diets rich in meat are claimed to contribute to the high tissue arachidonic acid (20:4ω6) content in people in Westernized societies, but there are very few direct data to substantiate this assertion. Because meat contains a variety of long-chain polyunsaturated fatty acids (PUFA) that are susceptible to oxidation, we initially examined the effect of cooking on the long-chain PUFA content of beef, and then determined the effect of ingestion of lean beef on the concentration of long-chain PUFA in plasma phospholipids (PL). First, we examined the effect of grilling (5–15 min) and frying (10 min) different cuts of fat-trimmed lean beef on the long-chain PUFA content. Second, we investigated the effect of including 500 g lean beef daily (raw weight) for 4 wk on the fatty acid content and composition of plasma PL in 33 healthy volunteers. This study was part of a larger trial investigating the effect of lean beef on plasma cholesterol levels. In the first two weeks, the subjects ate a very low-fat diet (10% energy) followed by an increase in the dietary fat by 10% each week for the next 2 wk. The added fat consisted of beef fat, or olive oil (as the oil or a margarine) or safflower oil (as the oil or a margarine). This quantity of beef provided 60, 230, 125, 140 and 20 mg/d, respectively, of eicosatrienoic acid (20:3ω6), 20:4ω6, eicosapentaenoic acid (20:5ω3), docosapentaenoic acid (22:5ω3) and docosahexaenoic acid (22:6ω3). Grilling for 10–15 min, but not frying, of the fat-trimmed lean beef resulted in 20–30% losses of the 20 and 22 carbon PUFA. The consumption of the lean beef during the first two-week period, when there was a very low level of dietary fat, was associated with significant increases in the proportion and concentration of 20:3ω6, 20:4ω6, 20:5ω3 and 22:5ω3 in the plasma PL and a significant decrease in the proportion and content of 18:2ω6. The addition of beef fat or olive oil to the diets containing lean beef did not alter the plasma PL fatty acid profile compared with the very low-fat diet, whereas the addition of safflower oil maintained the significant increases in 20:4ω6 and 22:5ω3 but led to decreases in 18:3ω3 and 20:5ω3 compared with the very low-fat diet. The results showed that diets rich in lean beef increased the 20:3ω6, 20:4ω6 and the long-chain ω3 PUFA levels in the plasma PL. A high level of linoleic acid in diets rich in lean beef prevented the rise in the plasma level of 20:3ω6 and 20:5ω3, two fatty acids known to antagonize the effects of 20:4ω6 on platelet aggregation.
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Following the intensive research in the 1980s on the role of ω6 and ω3 polyunsaturated fatty acids (PUFA) in human nutrition, there is now general agreement that the ratio of the ω6 to ω3 PUFA is too high in the current Western diet (1). This dietary imbalance is known to cause an imbalance in the ratio of ω6 to ω3 PUFA in tissues and a consequent exaggerated metabolism of arachidonic acid (20:4ω6) to eicosanoids (2).

The main PUFA in the Western diet is linoleic acid (18:2ω6), with lesser proportions of other PUFA such as α-linolenic acid (18:3ω3), arachidonic acid (20:4ω6), eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3). The intake of 18:2ω6 is of the order of 10–20 g/d, and this constitutes more than 85% of total PUFA intake (3,4). The high level of ω6 PUFA in tissues is a result of the dominance of the current food supply by 18:2ω6, which is the precursor of 20:4ω6, which, in turn, is the substrate for the eicosanoids. The fatty acid 18:3ω3 can compete with 18:2ω6 for metabolism *via* the desaturation and chain elongation enzymes (5), but because the dietary intake of this PUFA is low there is little restraint on the production of 20:4ω6.

Another source of tissue 20:4ω6 is that which originates directly from the diet, from foods such as meat, organ meats, eggs and fish (6). It is known that dietary 20:4ω6 is efficiently incorporated into tissue lipids in animals and humans (7–10), although there are very low levels of 20:4ω6 in the diet relative to linoleic acid.

There is little information on the average daily intake of 20:4ω6 in typical Western diets, and data for the content of this fatty acid in specific foods are incomplete. Phinney *et al.* (11) have suggested that, for U.S. adults eating eggs and meat, the intake is probably in the range 200–1000 mg/d. This agrees with the figure of about 500 mg/d determined on a mixed food omnivorous diet by Garg *et al.* (12). The 1985 Victorian Nutrition Survey reported 20:4ω6 intakes of 0.4–0.5 g/d based on a semi-quantitative food frequency questionnaire that used the British food tables as the nutrient data base, with additional data for some Australian foods (4). Based on a 24-hour dietary recall method and using Food Tables from the University of Minnesota, Dolecek (3) estimated the 20:4ω6 level in the MRFIT study at about 200 mg/d.

Diets rich in meat are claimed to contribute to the high tissue 20:4ω6 content in people in Westernized societies (13,14), but there are very few direct data to substantiate this assertion. Phinney *et al.* (11) have reported that omnivores with a relatively high intake of meat had a higher plasma 20:4ω6 level than did vegetarians. Sinclair *et al.* (10) reported that diets rich in kangaroo meat (100 mg 20:4ω6/100 g meat) induced higher plasma 20:4ω6 levels than did vegetarian diets.

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Abbreviations: PGE_1, prostaglandin E_1; PL, phospholipids; P/S, polyunsaturated fatty acid/saturated fatty acid ratio; PUFA, polyunsaturated fatty acids; TXA_2, thromboxane A_2.

The present study was designed to test whether diets rich in lean beef were associated with increases in plasma 20:4ω6 levels. This study is part of a larger trial investigating the effect of lean beef on plasma cholesterol levels, the results of which have been reported previously (15,16). Because meat contains a variety of long-chain PUFA that are susceptible to oxidation, we initially examined the effect of cooking on the long-chain PUFA content of beef, and then determined the effect of ingestion of lean beef (500 g/d) on the concentration of long-chain PUFA in plasma phospholipids (PL) using capillary gas chromatography.

MATERIALS AND METHODS

The meat for the dietary study and the fatty acid analyses was obtained from commercial butcher shops at the Queen Victoria Market, Melbourne, Australia and from retail outlets in Geelong, Australia. The subjects for the study were recruited through the Department of Medicine, Royal Melbourne Hospital and the Department of Human Nutrition, Deakin University, Geelong, as described previously (15,16). The protocols for the beef plus beef fat study and the beef plus olive oil or safflower oil studies were approved by the Ethics Committees of the Royal Melbourne Hospital and Deakin University, respectively.

Subjects. Volunteers for these studies were thirty-three free living, weight stable subjects (16 men, 17 women) with plasma cholesterol levels below 6 mM. In Study 1 (lean beef plus beef fat), the mean age and body weight of the 11 subjects (5 males and 6 females) were 25 ± 2 yr and 70 ± 3 kg (mean ± SD), respectively. In Study 2 (lean beef plus olive oil), the mean age and weight of the 11 subjects were 47 ± 14 yr and 71 ± 9 kg, respectively (for 5 males and 6 females), and in Study 3 (lean beef plus safflower oil), the mean age and weight of the 11 subjects were 38 ± 11 yr and 73 ± 15 kg, respectively (for 6 males and 5 females).

Diets. The details of the diets have been published previously together with the results on the effect of the diets on the plasma lipoprotein cholesterol and triglyceride composition (15,16). There were three studies, each lasting 5 wk with subjects acting as their own controls. The subjects weighed and recorded all food and liquid intake for the duration of the study. The first week was a baseline period in which subjects consumed their habitual diets containing their usual intake of 30–100 g beef per day. In the second and third weeks, the subjects consumed a very low-fat diet (10% energy) derived from vegetables, fruit, cereals, and nonfat dairy products, together with 500 g per day (raw weight) of fat-trimmed lean beef. If the subject's usual daily energy intake was less than 1800 kcal, the daily beef allowance was reduced to 400 g. The lean beef was supplied to the subjects in fat-trimmed preweighed packages daily, and it consisted of topside, rump, minced topside and diced steak. Samples were taken regularly for total fat analysis with the mean (±SD) fat content being 4.9 ± 0.9 g/100 g wet weight (n = 24). In the fourth and fifth weeks, the subjects continued to consume the lean beef, but the fat content of the diet was increased 10% energy per week so that in week 4 the total fat content was 20% of energy, and in week 5 it was 30% of energy. In Study 1, the fat was provided in the form of beef fat, in Study 2 the fat was olive oil or an olive oil-based margarine and in Study 3 the fat was safflower oil or a safflower oil-based margarine. The energy intake was maintained constant throughout each study. Because of the low-energy density and high bulk of the diet in weeks 2 and 3, the subjects were provided with a carbohydrate supplement (a glucose polymer; Polycose, Ross Laboratories, Columbus, OH) that was equivalent to 20% of energy in weeks 2 and 3. In weeks 4 and 5, the intake of the carbohydrate supplement was reduced as the fat was increased, thus ensuring a constant energy intake. As reported previously (15,16), the fat intake of all 33 subjects on their usual diets was 36.6% of total energy with a ratio of polyunsaturated to saturated fatty acids (P/S) of 0.38. On the very low-fat diet containing lean beef, the dietary fat contributed 9.3% dietary energy with a P/S ratio of 0.49. After two weeks on the lean beef plus beef fat diet (week 5 of Study 1), the dietary fat level was 29.1% dietary energy with a P/S of 0.21. On the lean beef plus olive oil diet, the total fat intake was 29.6% energy with a P/S of 0.63, and on the lean beef plus safflower oil diet the fat intake was 27.9% energy with a P/S of 2.89. The long-chain PUFA content of the beef was based on the analysis of samples of lean beef collected from the same outlets used to supply the beef consumed during the studies. The analytical procedures used are described below. The 500 g lean beef contained the following amounts of 20:3ω6, 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3 (in mg): 60, 230, 125, 140, 20, respectively. There were no other dietary sources of these fatty acids during the 4-week period of beef consumption.

Analytical methods. To determine the effect of cooking on the fatty acid content of lean beef, five pieces of rump steak (approx. 800 g) were purchased from five separate commercial outlets. The visible fat was trimmed from the steak, and it was then divided into six portions each of about 100 g. One portion of steak from each of the five pieces was grilled in a gas stove for 15 min (well-done), one for 10 min (medium), one for 5 min (rare) and one left uncooked. An additional piece was fried without added fat or oil in a Teflon-coated frying pan for 10 min, and the remaining piece was minced and then fried as above for 5 min. After cooking, the steaks were allowed to cool on a piece of absorbent paper towel, the meat was minced and a 10 g sample was extracted in chloroform/methanol (2:1, vol/vol) containing antioxidant as previously described (17). The moisture content was determined on a 2 g sample by heating in an oven at 105°C for 24 h. The total lipid content was determined gravimetrically, and on a separate aliquot the methyl esters of the total fatty acids were formed by saponification in KOH in methanol followed by esterification with BF_3 in methanol (17). The fatty acid methyl esters were separated by capillary gas–liquid chromatography using a 50-m × 0.32-mm i.d. fused silica column coated with CP Sil 88 (Chrompak, Middleburg, The Netherlands), and the amounts were quantified using heptadecanoic acid as an internal standard (17). The cholesterol content was determined by capillary gas–liquid

chromatography using a 25-m × 0.22-mm i.d. fused silica bonded-phase column (BP-1) (S.G.E., Melbourne, Australia). Dihydrocholesterol (5α-cholestanol) was used as an internal standard. Aliquots of the total lipid plus 5α-cholestanol were saponified, extracted and then derivatized with *bis*(trimethylsilyl)trifluoroacetamide in pyridine (Pierce Chemical Company, Rockford, IL) for 30 min at room temperature.

Fasting blood samples (10 mL into heparinized tubes) were obtained from the subjects at weekly intervals throughout the study. The plasma was stored at —20°C until analyzed. Lipids were extracted from the plasma using chloroform/methanol (2:1, vol/vol), and the phospholipids were separated from the neutral lipids by thin-layer chromatography (10). A known amount of diheptadecanoyl phosphatidylcholine (internal standard) was added to the plasma prior to extraction. The methyl esters of the PL fatty acids were formed by a saponification step followed by transesterification in BF_3 in methanol (17), and the fatty acid methyl esters were separated by capillary gas–liquid chromatography as described above.

Statistics. The paired *t*-test was used to compare results within groups. Results are expressed as mean ± SEM; significance was taken as $P < 0.05$.

RESULTS

The lean, fat-trimmed raw beef contained about 4.3 g of total lipid, including 49 mg cholesterol, and 74.3 g of water per 100 g fresh weight. Cooking decreased the moisture content of all samples. When this loss of moisture was considered, there was no significant decrease in either total lipid or cholesterol (Table 1). The main PUFA in the samples were (in decreasing order of concentration): 18:2ω6, 20:4ω6, 20:5ω3, 22:5ω3, 18:3ω3, 20:3ω6 and 22:6ω3. The 20:4ω6 and 20:5ω3 concentrations of the fat-trimmed lean beef were 37 and 23 mg/100 g wet weight, respectively. There were significant ($P < 0.05$) decreases in the contents of all the 20 and 22 carbon PUFA in the grilled beef compared with the raw samples, although there was no consistent loss of a specific PUFA as the length of cooking increased. Frying minced or whole steaks did not lead to significant losses of the PUFA, apart from a significant decrease in 22:5ω3 in the minced steak (Table 2). There were no significant changes in the concentration of the major saturated or monounsaturated fatty acids following cooking.

TABLE 1

The Effect of Cooking on the Total Lipid and Cholesterol Content of Lean Beef[a]

Beef sample	Cooking technique	Total lipid (g)	Cholesterol (mg)
Raw		4.3 ± 0.7	49 ± 2
Rare	Grilled	3.3 ± 0.3	50 ± 2
Medium		3.6 ± 0.4	52 ± 2
Well-done		3.6 ± 0.2	51 ± 1
Minced		3.5 ± 0.3	50 ± 1
Fried	Pan-fried	3.4 ± 0.2	50 ± 1

[a]Results are expressed per 100 g equivalent fresh weight, mean ± SEM. There were five samples analyzed for each cooking method. The results for the cooked samples are expressed per 100 g equivalent raw sample, i.e., adjusted for the moisture loss on cooking (see Materials and Methods). There were no significant differences between the cooked samples and the raw sample.

Significant changes in the plasma PL fatty acid composition of the thirty-three subjects occurred after they had consumed a very low-fat diet containing 500 g/d of fat-trimmed lean beef for 2 wk. The effect of this dietary change on the fatty acid composition of the plasma PL is shown in Table 3 and Figure 1. The proportion of 18:2ω6 decreased ($P < 0.001$), while that of the other ω6 PUFA increased significantly, namely 20:3ω6 ($P < 0.001$), 20:4ω6 ($P < 0.001$), 22:4ω6 ($P < 0.001$) and 22:5ω6 ($P < 0.001$). The proportions of 20:5ω3 ($P < 0.001$) and 22:5ω3 ($P < 0.001$) also increased. The proportion of 18:0 decreased $P < 0.05$) while that of 16:0, 16:1 and 18:1 increased ($P <$ 0.001). There were also significant elevations, in plasma PL, in all 20 and 22 carbon PUFA levels except 22:6ω3. The increases in the long-chain PUFA were 11 mg/L for 20:3ω3 ($P < 0.001$), 28 mg/L for 20:4ω6 ($P < 0.001$), 5 mg/L for 20:5ω3 ($P < 0.001$) and 7 mg/L for 22:5ω3 ($P < 0.001$). There were also statistically significant changes in the proportions of the plasma PL PUFA one week after commencing the very low-fat diet (data not shown).

TABLE 2

The Effect of Cooking on the Mean Content of Polyunsaturated Fatty Acids in Fat-Trimmed Lean Beef[a]

Sample	Treatment	18:2ω6	18:3ω3	20:3ω6	20:4ω6	20:5ω3	22:5ω3	22:6ω3
Raw	—	61 ± 9	17 ± 2	10 ± 1	37 ± 5	23 ± 2	23 ± 0.4	3 ± 0.4
Rare	Grilled	60 ± 6	18 ± 2	8 ± 1[b]	32 ± 3	20 ± 2	21 ± 0.4[c]	3 ± 0.3
Medium	Grilled	54 ± 5	16 ± 1	8 ± 1[b]	30 ± 3[c]	18 ± 2[d]	18 ± 2[c]	2 ± 0.3[c]
Well-done	Grilled	57 ± 6	17 ± 1	8 ± 1[b]	29 ± 3[c]	18 ± 1[c]	19 ± 1[c]	2 ± 0.2[b]
Minced	Fried	63 ± 7	19 ± 2	8 ± 1[b]	32 ± 4	21 ± 2	21 ± 1[b]	3 ± 0.4
Medium	Fried	54 ± 7	16 ± 2	9 ± 1	32 ± 3	22 ± 2	22 ± 2	3 ± 0.4

[a]The results for the five samples are expressed as mg per 100 g raw sample, mean ± SEM. For the cooked samples, the results are given per 100 g equivalent raw sample (i.e., adjusted for the moisture loss on cooking).

[b–d]Significantly different from raw sample (paired *t*-test), [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$.

TABLE 3

Fatty Acid Composition of Plasma Phospholipids over the Five-Week Intervention in Subjects Fed a Very Low-Fat Diet Rich in Lean Beef in Weeks 2–3 and Then Supplemented with Beef Fat, Olive Oil or Safflower Oil in Weeks 4–5[a]

	Week 1 (usual diet)	Week 3 (very low-fat)	Week 1 (usual diet)	Week 5 (+beef fat)	Week 1 (usual diet)	Week 5 (+olive oil)	Week 1 (usual diet)	Week 5 (+safflower oil)
Fatty acid	(n = 33)	(n = 33)	(n = 11)	(n = 11)	(n = 11)	(n = 11)	(n = 11)	(n = 11)
				(g/100 g fatty acids)				
14:0	0.41 ± 0.02	0.37 ± 0.03	0.38 ± 0.03	0.32 ± 0.02	0.42 ± 0.04	0.31 ± 0.03[b]	0.44 ± 0.05	0.35 ± 0.05
16:0	27.56 ± 0.42	29.06 ± 0.43[d]	26.22 ± 0.58	27.14 ± 0.98	28.63 ± 0.64	27.15 ± 0.94[c]	27.84 ± 0.86	26.60 ± 0.89
18:0	11.89 ± 0.27	11.21 ± 0.32[b]	12.57 ± 0.42	11.99 ± 0.65	11.68 ± 0.57	10.51 ± 0.70	11.41 ± 0.40	10.02 ± 0.88[b]
16:1	1.69 ± 0.27	1.83 ± 0.09[d]	2.17 ± 0.82	1.86 ± 0.09[d]	1.44 ± 0.10	1.41 ± 0.09	1.47 ± 0.15	1.31 ± 0.16
18:1	11.75 ± 0.29	13.16 ± 0.25[d]	12.80 ± 0.48	15.69 ± 0.77[c]	10.77 ± 0.39	13.75 ± 0.39[c]	11.68 ± 0.50	8.06 ± 0.36[d]
18:2ω6	24.61 ± 0.57	16.53 ± 0.47[d]	24.44 ± 0.84	18.92 ± 1.15[d]	24.55 ± 1.29	20.07 ± 8.93[c]	24.85 ± 0.94	28.29 ± 1.04[d]
20:2ω6	0.32 ± 0.02	0.34 ± 0.02	0.37 ± 0.04	0.46 ± 0.06	0.30 ± 0.02	0.26 ± 0.02	0.29 ± 0.03	0.36 ± 0.03
20:3ω6	3.05 ± 0.12	4.14 ± 0.13[d]	3.29 ± 0.25	3.80 ± 0.23[b]	2.83 ± 0.22	3.33 ± 0.21[c]	3.05 ± 0.19	3.16 ± 0.28
20:4ω6	11.28 ± 0.36	14.08 ± 0.41[d]	10.56 ± 0.61	13.34 ± 0.90[c]	11.97 ± 0.52	14.61 ± 0.67[d]	11.30 ± 0.78	14.80 ± 1.12[d]
22:4ω6	0.33 ± 0.02	0.45 ± 0.02[d]	0.39 ± 0.04	0.45 ± 0.04	0.32 ± 0.02	0.31 ± 0.02	0.29 ± 0.04	0.35 ± 0.04[d]
22:5ω6	0.27 ± 0.02	0.34 ± 0.02[d]	0.33 ± 0.04	0.37 ± 0.33	0.25 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.25 ± 0.03
Total ω6	39.87 ± 0.53	35.88 ± 0.47[d]	39.38 ± 0.62	37.34 ± 1.80	40.22 ± 1.16	38.83 ± 1.19	40.02 ± 1.04	47.23 ± 1.33[d]
18:3ω3	0.28 ± 0.02	0.26 ± 0.02	0.34 ± 0.04	0.33 ± 0.04	0.23 ± 0.02	0.19 ± 0.02	0.26 ± 0.03	0.15 ± 0.02[c]
20:5ω3	1.35 ± 0.10	1.76 ± 0.10[d]	1.39 ± 0.18	1.98 ± 0.15[c]	1.16 ± 0.15	1.73 ± 0.24[d]	1.50 ± 0.22	1.06 ± 0.12[b]
22:5ω3	1.20 ± 0.06	1.80 ± 0.08[d]	1.47 ± 0.11	1.91 ± 0.13[d]	1.03 ± 0.08	1.47 ± 0.09[d]	1.09 ± 0.11	1.34 ± 0.10[d]
22:6ω3	4.53 ± 0.22	4.88 ± 0.02	4.93 ± 0.30	5.07 ± 0.47	4.35 ± 0.32	4.45 ± 0.30	4.31 ± 0.52	3.91 ± 0.38
Total ω3	7.35 ± 0.33	8.70 ± 0.26[c]	8.13 ± 0.40	9.29 ± 0.53[d]	6.77 ± 0.48	7.85 ± 0.47	7.17 ± 0.77	6.46 ± 0.53

[a]Mean ± SEM. [b–d]Significantly different from week 1: [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$.

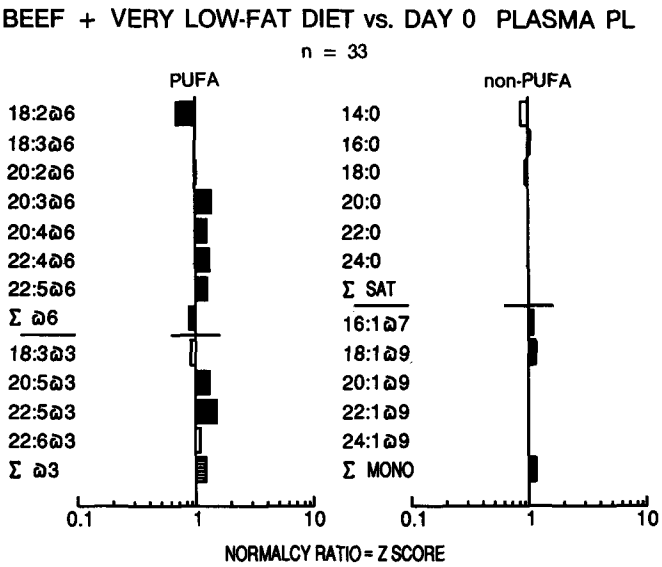

FIG. 1. Plasma phospholipid (PL) fatty acid profile expressed as a normalcy ratio for subjects on very low-fat diet rich in lean beef compared with the fatty acid profile on the subject's usual high-fat diet. The normalcy ratio is the value for a fatty acid on the very low-fat diet divided by the value on the usual diet (see Ref. 31). The black bars indicate $P < 0.001$; closely striated bars, $P < 0.01$; widely striated bars, $P < 0.05$; and open bars nonsignificant change. PUFA, polyunsaturated fatty acids; SAT, saturated; MONO, monounsaturated.

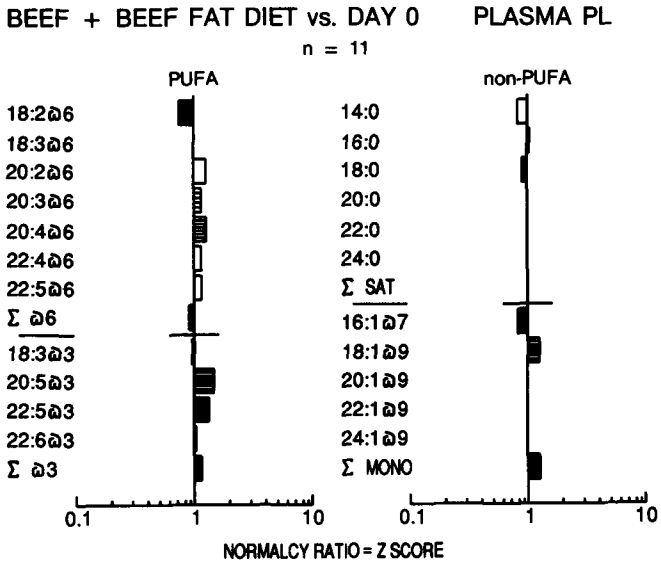

FIG. 2. Plasma PL fatty acid profile expressed as normalcy ratio for subjects on a high-fat diet rich in beef fat plus lean beef compared with the fatty acid profile on the subject's usual high-fat diet. Significant differences and abbreviations as in Figure 1.

The addition of fat to the very low-fat diet containing lean beef was associated with significant changes in the plasma PL fatty acids that were dependent upon the nature of the fat included in the diet (Table 3 and Figs. 2–4). Beef fat plus lean beef, and olive oil plus lean beef, were associated with significant increases in the percentage of 18:1, 20:3ω6, 20:4ω6, 20:5ω3 and 22:5ω3 and a significant decrease in 18:2ω6 compared with the values obtained when the subjects were on their usual diets. In contrast, safflower oil plus lean beef led to a significant decrease in oleic acid, 18:3ω3 and 20:5ω3 and an increase in 18:2ω6, 20:2ω6, 20:4ω6, 22:4ω6 and 22:5ω3.

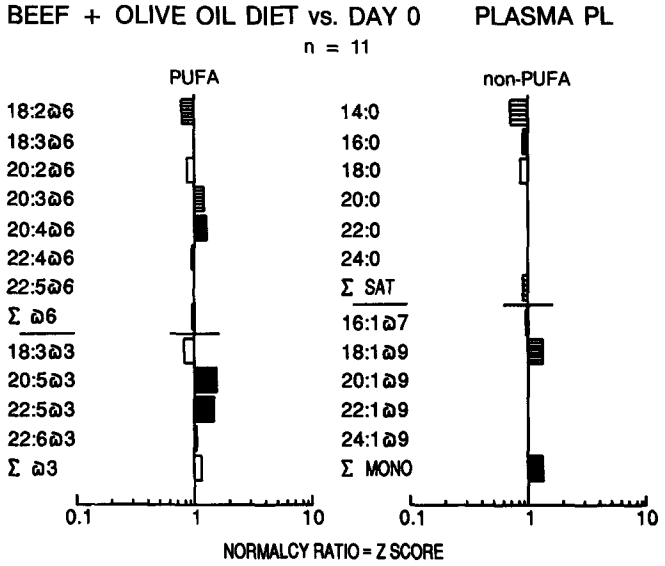

FIG. 3. Plasma PL fatty acid profile expressed as normalcy ratio for subjects on a high-fat diet rich in olive oil plus lean beef compared with the fatty acid profile on the subject's usual high-fat diet. Significant differences and abbreviations as in Figure 1.

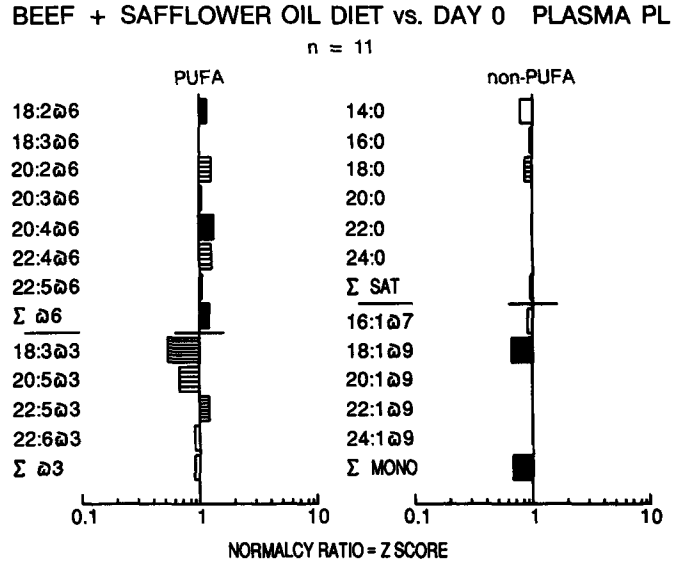

FIG. 4. Plasma PL fatty acid profile expressed as normalcy ratio for subjects on a high-fat diet rich in safflower oil plus lean beef compared with the fatty acid profile on the subject's usual high-fat diet. Significant differences and abbreviations as in Figure 1.

This dietary study was also designed to determine the effect of inclusion of lean beef on plasma lipoprotein lipid concentrations. The results have been reported previously (15,16), and they indicated that the inclusion of 500 g lean beef daily in the very low-fat diet was associated with a significant decline in total plasma cholesterol concentration ($P < 0.001$) compared with the subject's usual diet. This effect was maintained when either olive or safflower oils were added back to this diet ($P < 0.001$); however, the addition of beef fat was associated with a significant elevation in the total plasma cholesterol level ($P < 0.01$).

DISCUSSION

The first purpose of this study was to determine the effect of cooking on the long-chain PUFA content of beef. Although the USDA Handbook on the composition of meat lists 20:4ω6 as the only long-chain PUFA in a variety of beef cuts (18), there have been recent reports from the United States and Australia, based on analyses conducted using capillary gas-chromatographic columns, of significant levels of 20:3ω6, 20:4ω6, 20:5ω3 and 22:5ω3 in pasture and grain-fed beef (17,19). There were significant losses of some PUFA with increasing time of grilling, particularly the 20 and 22 carbon PUFA, in which case the losses amounted to 20–33%. Previous studies on the effect of cooking on the fatty acid composition of the intramuscular PL or total lipids found no difference between the proportion of 18-carbon PUFA in raw and cooked samples of beef, although the most highly unsaturated fatty acids (20 and 22 carbon ω3 PUFA) were not determined in those studies (20–22). The losses of the more highly unsaturated fatty acids are not surprising since they are more susceptible to oxidation than the 18-carbon PUFA (23).

The second purpose of this study was to learn whether long-chain PUFA in beef can make a significant contribution to plasma PL fatty acids of subjects consuming 500 g/d of lean beef. The inclusion of lean beef in the diet of the subjects was associated with significant rises in the proportion and concentration of 20:3ω6, 20:4ω6, 20:5ω3 and 22:5ω3 and a significant decline in 18:2ω6 in the plasma PL. We ascribe these changes to the meat in the diet rather than the change to the very low-fat diet because these fatty acids were present in the lean beef, and we have previously shown that similar very low-fat vegetarian diets did not lead to increases in these PUFA in plasma PL (10). These fatty acids were all present in the lean beef, even though the levels were as low as 50 mg/d (from 500 g raw beef) in the case of the 20:3ω6. There was no increase in either 18:3ω3 or 22:6ω3 in the plasma PL although these two fatty acids were also present in the beef. The 22:6ω3 was found in the lowest concentration (20 mg/500 g beef), whereas the 18:3ω3 was present at a higher level (85 mg/500 g beef). The 18:3ω3 may have partitioned into the plasma triglyceride fraction, or it may have been preferentially metabolized *via* oxidation or conversion to 20:5ω3. The former two possibilities may be more likely explanations (24).

The addition of dietary fat to the very low-fat beef diet was associated with fat-specific changes in the plasma PL fatty acid profile. Both beef fat and olive oil added at a level of 20% energy led to increases in 20:3ω6, 20:4ω6, 20:5ω3 and 22:5ω3. The same changes were observed in the plasma PL on the very low-fat diet. In contrast, the addition of 20% energy from safflower oil was associated with rises in all the ω6 PUFA (except 20:3ω6) and in 22:5ω3 and significant decreases in 18:3ω3 and 20:5ω3 in the plasma PL. These results show that the three diets with the lowest 18:2ω6 levels (very low-fat diet, beef plus

beef fat diet and beef plus olive oil diet; all less than 5% energy as 18:2ω6) had similar effects on the fatty acid profile of the plasma PL. This suggests that at very high 18:2ω6 levels (beef plus safflower oil >14% of energy), the 18:2ω6 reduces the metabolism of 18:3ω3 to 20:5ω3 and/or competes more effectively with 18:3ω3 and 20:5ω3 in the acylation into PL. High dietary 18:2ω6 levels have previously been reported to result in low 20:5ω3 levels in the serum PL fraction in a five-month dietary study (25).

The results from this study show that a variety of long-chain ω6 and ω3 PUFA found in lean beef, not just 20:4ω6, contribute to the pool of these PUFA found in plasma PL. However, the effects of beef on the plasma PL of humans are modulated by the level of 18:2ω6 in the diet. If the diet contains lean beef and a modest to low 18:2ω6 level, the effect on the plasma PUFA profile is an increase in 20:3ω6, 20:4ω6 and 20:5ω3. In contrast, if the diet containing lean beef is also rich in 18:2ω6, only increases in 18:2ω6 and 20:4ω6 in plasma PL are observed. We recognize that the amount of lean beef consumed in this study was high relative to the usual intakes of lean meat in this country (4). However, we have preliminary evidence that lower intakes of lean beef (300 g/d) also result in significant increases in the proportion of long-chain PUFA in plasma PL in human subjects (Sinclair, A.J., and Morgan, S.A., unpublished data).

There is a concern that diets that raise 20:4ω6 might be prothrombotic (13,14,26), and meat has been viewed as a major source of dietary 20:4ω6. The present results show that this view may be too simplistic. We acknowledge that the changes described in the proportion of plasma PL PUFA in this study were quite small. However, we have preliminary evidence that diets rich in lean red meat, which induced similar changes in 20 carbon PUFA in the plasma PL of seven subjects, were associated with significant reductions in the *in vivo* production of prostacyclin (as measured by gas chromatography/mass spectrometry of the main urinary metabolite; Mann, N.J., and Sinclair, A.J., unpublished data). We have not yet determined the effect of this diet on thromboxane production.

We propose that diets that increase the proportion of 20:3ω6, 20:4ω6 and 20:5ω3 in plasma (and presumably tissue) PL might not have the same effect on thrombosis as diets that raise 20:4ω6 alone, for the following reasons. Increased levels of plasma 20:4ω6 may lead to increases in platelet 20:4ω6 that upon appropriate stimulation could lead to the production of thromboxane A_2 (TXA_2) with resultant platelet aggregation (9). Increased levels of 20:3ω6 and 20:5ω3 might be potentially antithrombotic since the former is a precursor of prostaglandin E_1 (PGE_1) that prevents platelet aggregation (27), and the latter (20:5ω3) has been shown to effectively reduce production of TXA_2 by platelets and platelet aggregation (28). Thus, raised levels of all three eicosanoid precursors (20:3ω6, 20:4ω6 and 20:5ω3) may result in a different outcome in terms of eicosanoid production and platelet aggregation. It may be possible to substantiate this hypothesis by determining the effect of diets rich in lean beef on platelet fatty acid composition, platelet aggregation and the analysis of the main urinary metabolites of thromboxane and prostacyclin (29) in humans.

The importance of a balance of dietary ω3 to ω6 fatty acids is becoming recognized gradually, although the competition between these two families of PUFA has been known for decades (5). The cholesterol-lowering properties of linoleic acid-containing oils have been known even longer, and the modern food industry has developed and now produces several stable linoleate-rich oils. Linolenic acid oils have been avoided as food fats, largely because of the unsavory oxidation products of linolenic acid. The result of these developments has been to induce a very low ratio of ω3/ω6 fatty acids in the Western diet on a wide scale. The extreme situation of ω3 deficiency has been observed in a child with accompanying neurological disturbances (30). Recent studies of the PUFA profile in plasma have revealed that many diseases of the modern Western world are accompanied by suppression of total ω3 PUFA and total ω6 PUFA when compared with healthy controls, as well as a lessened fluidity of the plasma PL (31).

It is tempting to correct the low ω3 profiles by supplements of fish oils rich in long-chain ω3 PUFA, but this strategy, imposed in the context of a typical Western diet rich in 18:2ω6, can lead to profiles rich in 18:2ω6 and the long-chain ω3 PUFA and a suppression of the long-chain ω6 PUFA such as 20:3ω6, 20:4ω6 and 22:4ω6. The beneficial effects of lean beef in enhancing the plasma PL status of these PUFA should be emphasized and could be utilized without concern of hypercholesterolemia, since the diets low in saturated fat and rich in lean beef used in this study actually lowered circulating cholesterol levels (15,16).

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The Influence of Vitamin E and Selenium on Lipid Peroxidation and Aldehyde Dehydrogenase Activity in Rat Liver and Tissue

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Malondialdehyde (MDA) production and cytosolic aldehyde dehydrogenase (ALDH) response were examined in rat liver tissues after feeding different levels of dietary vitamin E and/or selenium and polyunsaturated fat for 12–38 wk. MDA production was significantly increased by vitamin E deficiency or by high levels of polyunsaturated fat intake, but not by selenium deficiency. The activity of cytosolic ALDH increased upon increased production of MDA after 12–16 wk of feeding the lipid peroxidation-inducing diets. However, ALDH activity was suppressed after 38 wk of feeding the vitamin E-deficient diet. The results indicate that the hepatic cytosolic ALDH may be involved in the metabolism of MDA during a relatively short-term increase in *in vivo* lipid peroxidation, but that ALDH activity becomes suppressed after more severe *in vivo* lipid peroxidation has been produced. Hepatic and plasma α-tocopherol levels and lipid peroxidation products were measured for the various dietary groups. *Lipids* 29, 345–350 (1994).

Malondialdehyde (MDA) is a highly reactive and cytotoxic aldehyde and is one of the major products formed in the course of peroxidative degradation of membrane lipids. It has been assumed that the MDA released from mitochondrial and microsomal membranes during polyunsaturated fatty acid (PUFA) peroxidation is metabolized by hepatocytes. Mammalian liver contains a variety of pyridine nucleotide-linked aldehyde dehydrogenases (ALDH; EC 1.2.1.3) that possess wide substrate specificities and are widely distributed among the mitochondrial, microsomal and cytosolic fractions (1–3).

Early investigators (4–6) had concluded that MDA is predominantly oxidized by the mitochondrial fraction of rat liver. By contrast, Hjelle and Petersen (7) more recently suggested that the capacity to metabolize MDA is greatest in rat liver cytosol rather than in mitochondria. Further work by Hjelle and Petersen (8) provided evidence, based on *in vitro* kinetic properties, that the cytosolic ALDH isozyme may in fact predominate in MDA metabolism. This concept is also consistent with the findings of Pirozhkov and Panchenko (9), Marselos and Vasiliou (10) and Vasiliou and Marselos (11). However, because these studies were done on isolated tissues and tissue subfractions, direct information concerning the involvement of cytosolic ALDH in the *in vivo* metabolism of MDA could not be obtained.

In the present study, *in vivo* lipid peroxidation was experimentally induced by administering diets deficient in vitamin E or selenium (Se), or both, and by diets high in PUFA. The objective of the study was to determine the extent of hepatic MDA accumulation induced by the treatments. In addition, hepatic and plasma α-tocopherol levels and lipid peroxidation products were determined. Because hepatic ALDH may play a role in MDA metabolism, changes in the activity of the low affinity cytosolic form of this enzyme induced by the various diets were also measured.

MATERIALS AND METHODS

Induction of in vivo *lipid peroxidation.* Three different animal feeding protocols were used to induce *in vivo* lipid peroxidation.

Experiment 1. In vivo lipid peroxidation was induced by long-term vitamin E deficiency. For 38 wk, one group of 10 female weanling Sprague-Dawley rats was fed a modified basal vitamin E-deficient diet containing 0.05 ppm Se, as described by Draper *et al.* (12), which was adequate in all respects except for vitamin E (–E), while a second group was fed a basal diet supplemented with 30 ppm vitamin E (as *RRR*-α-tocopheryl acetate; 1.36 IU/mg, Type 3; Sigma Chemical Company, St. Louis, MO) (NE). Diet and water were provided *ad libitum.*

Experiment 2. In vivo lipid peroxidation was modulated by administering three levels of dietary PUFA. Groups of 10 female weanling Sprague-Dawley rats were fed either a low- (LP), medium- (MP) or high- (HP) PUFA diet containing 30 ppm vitamin E for 16 wk. The composition of the diets is shown in Table 1. The content of dietary fat was 15%, and the degree of unsaturation of dietary fat was adjusted by using combinations of lard, corn oil and cod liver oil.

Experiment 3. In vivo lipid peroxidation was induced by deficiencies in Se, vitamin E, or both. Four groups of 10 female weanling Sprague-Dawley rats were fed one of the following diets for 12 wk: (i) a Torula yeast-based Se- and vitamin E-deficient basal diet, as described by Schwartz and Fredga (13) (see Table 2), for the double deficient group (–Se–E); (ii) a basal diet supplemented with 30 ppm vitamin E as *RRR*-α-tocopheryl acetate for the Se-deficient group (–Se+E); (iii) a basal diet supplemented with 0.2 ppm Se as sodium selenite for the vitamin E-deficient group (+Se–E); or (iv) a basal diet supplemented with 30 ppm vitamin E and 0.2 ppm Se for the control group (+Se+E).

Measurement of hepatic MDA production. The extent of hepatic lipid peroxidation was measured by the thiobarbituric acid (TBA) test and by a high-performance liquid

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Abbreviations: ALDH, aldehyde dehydrogenase; –E, basal vitamin E-deficient diet; HP, high PUFA diet; LP, low PUFA diet; MDA, malondialdehyde; MP, medium PUFA diet; NE, diet supplemented with 30 ppm vitamin E; PUFA, polyunsaturated fatty acids; Se, selenium; –Se–E, Se- and vitamin E-deficient basal diet; –Se+E, diet supplemented with 30 ppm vitamin E as *RRR*-α-tocopheryl acetate for the Se-deficient group; +Se–E, diet supplemented with 0.2 ppm Se as sodium selenite for the vitamin E-deficient group; +Se+E, diet supplemented with 30 ppm vitamin E as *RRR*-α-tocopheryl acetate and 0.2 ppm Se; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

TABLE 1

Composition of Diets with Different Levels of Polyunsaturated Fatty Acids

Ingredients	Low PUFA (%)	Medium PUFA (%)	High PUFA (%)
Anhydrous d(+)-dextrose[a]	60.4	60.4	60.4
Vitamin-free casein[a]	20.0	20.0	20.0
Lard (stripped)[b]	15.0	10.0	
Corn oil (stripped)[b]		5.0	10.0
Cod liver oil[a]			5.0
Salt mix[c]	4.0	4.0	4.0
Vitamin mix[d]	0.5	0.5	0.5
Choline chloride[e]	0.1	0.1	0.1
Vitamin A[f]	10,000 IU/kg diet	10,000 IU	10,000 IU
Vitamin D_2[g]	1,000 IU/kg diet	1,000 IU	1,000 IU
Vitamin E[h]	30 mg/kg diet	30 mg	26.47 mg[i]

[a]United States Biochemical Corp., Cleveland, OH.
[b]Eastman Kodak Co., Rochester, NY.
[c]Salt mix 4164 (ICN Nutritional Biochemicals, Cleveland, OH).
[d]Vitamin Mix composition (g/kg mix); riboflavin 1.0, thiamine hydrochloride 2.0, nicotinic acid 5.0, pyridoxine hydrochloride 1.0, pantothenic acid (hemi-calcium salt) 2.0, menadione 0.2, folic acid 0.2, biotin 0.02 (all from Sigma Chemical Co., St. Louis, MO). Vitamin B_{12} (0.1% trituration with mannitol) 20.0 (ICN Nutritional Biochemicals), and d(+)-dextrose 968.58.
[e]Sigma Chemical Co.
[f]Vitamin A palmitate water dispersible (250,000 IU/g) (General Drug and Chemical Co., N. Kansas City, MO).
[g]Vitamin D_2 water dispersible (500,000 IU/g) from ICN Nutritional Biochemicals.
[h]*RRR*-α-tocopheryl acetate (Sigma Chemical Co.).
[i]From high-performance liquid chromatography analysis, 50 g cod liver oil/kg diet contained 2.52 mg of α-tocopherol which corresponds to 3.53 mg of α-tocopheryl acetate based on the biological activity. For this reason, the amount of α-tocopheryl acetate supplemented for the high polyunsaturated fatty acid (PUFA) diet was adjusted accordingly.

chromatography (HPLC) method. TBA reactive substances (TBARS) in liver tissues were measured by a modification of the method by Uchiyama and Mihara (14). TBARS in the samples were expressed as MDA equivalents. The amounts of free MDA produced in hepatic tissues were measured by the HPLC method developed by Csallany *et al.* (15). Details of the procedures have been described in a previous paper (16). MDA was prepared by hydrolysis of MDA tetramethyl acetal (Eastman Kodak, Rochester, NY) in 0.01N HCl for 1 h at 50°C. The hydrolyzed solution was titrated to pH 7.4 with 1N NaOH and then diluted with ice-cold 50 mM phosphate buffer (pH 7.4) to produce a 50 mM concentration of MDA. The concentration of MDA was then calculated from absorbance measurements at 267 nm based on the molar extinction coefficient of MDA (3.18×10^4 M^{-1} cm^{-1}).

TABLE 2

Composition of Selenium and Vitamin E-Deficient Basal Diets

Ingredients	%
Torula yeast[a]	30
Sucrose[b]	59
Stripped corn oil[c]	5
Salt mix[b,d]	5
Vitamin mix[b,e]	1

[a]United States Biochemical Corp., Cleveland, OH.
[b]ICN Nutritional Biochemicals, Cleveland, OH.
[c]Eastman Kodak Co., Rochester, NY.
[d]Hubbel, Mendel and Wakeman Salt mix.
[e]Vitamin mix composition (g/kg mix); Vitamin A acetate (500,000 IU/g) Vitamin D concentrate (850,000 IU/g) 0.125, ascorbic acid 45.0, inositol 5.0, niacin 4.25, riboflavin 1.0, pyridoxine hydrochloride 1.0, thiamine hydrochloride 1.0, calcium pantothenate 3.0, biotin 0.02, folic acid 0.090, vitamin B_{12} 0.00135, dextrose 855.46365.

Hepatic ALDH assay. Rats were deprived of food but had access to water *ad libitum* for 12 h before sacrifice. The rats were killed by decapitation, and the livers were removed immediately and rinsed thoroughly with ice-cold physiological saline. The fresh tissues were then homogenized in five volumes of ice-cold 1.15% KCl 10 mM phosphate buffer with 5 mM ethylenediamine tetraacetic acid tetrasodium salt (EDTA) (pH 7.4) using a Brinkman Polytron (Brinkman Instruments, Westbury, NY). The homogenates were centrifuged at 600 × *g* for 10 min and then at 9000 × *g* for 15 min in a Sorvall RC-5B refrigerated centrifuge (DuPont Instruments, Des Plaines, IL). The supernatants were subjected to further centrifugation on a Beckman L2-65B Ultracentrifuge (Beckman Instruments, Fullerton, CA) for 60 min at 105,000 × *g*. The resultant supernatant (cytosolic fraction) was used for the enzyme assay.

The enzyme activity was determined spectrophotometrically by monitoring absorbance increases at 340 nm caused by the reduction of NAD^+ to NADH. The molar extinction coefficient of NADH (6.3×10^3 M^{-1} cm^{-1}) was used to calculate the concentration of NADH (17). Reaction mixtures contained NAD (1 mM), pyrazole (2 mM), substrate (MDA, 5 mM), and enzyme in 50 mM phosphate buffer (pH 7.4). The absorbance changes were monitored

at 34°C for the initial 10 min using a Beckman DU-8 spectrophotometer (Beckman Instrument Co., Berkeley, CA) equipped with a Kinetics Compuset and were corrected for the absorbance changes of the blank (minus substrate). Because methanol is produced during the hydrolysis of MDA tetramethyl acetal, pyrazole was added to the reaction mixture to inhibit possible alcohol dehydrogenase-mediated NADH production.

The amount of protein in the cytosolic fraction was measured by the Lowry method (18) using bovine serum albumin as the standard.

Hepatic α-tocopherol determination. The concentrations of α-tocopherol in liver tissues were measured by the HPLC method developed by Zaspel and Csallany (19).

Measurement of plasma TBARS. Blood samples from the carotid artery of the rats were collected into 15-mL centrifuge tubes containing heparin. Plasma was separated by centrifuging blood samples at 478 × *g* for 15 min. Plasma TBARS were analyzed by a modified filtration procedure of Tarladgis *et al.* (20). Duplicate samples of 0.5 mL plasma were mixed thoroughly with 3 mL of 5% trichloroacetic acid and 1 mL of 0.06 M TBA solution in a screw-capped culture tube. The mixture was heated in an 80°C water bath for 90 min, cooled to room temperature, and then centrifuged at 478 × *g* for 15 min to remove the fine precipitate. The absorbance of the supernatant was read at 535 nm. Known amounts of standard MDA were treated in the same way. The amounts of plasma TBARS were expressed as MDA equivalents.

Statistical data analysis. The results were analyzed for their significance by Student's *t*-test in the comparison of two means, or by one-way analysis of variance followed by Tukey's multiple comparison test (21) in the comparisons of more than two means. Differences were considered significant when the *P* value was less than 0.05.

RESULTS AND DISCUSSION

Effect of dietary vitamin E on in vivo *lipid peroxidation.* The production of TBARS and free MDA and the specific activity of cytosolic ALDH in the long-term (38 wk) vitamin E-deficient and control rat liver tissues are shown in Figure 1. The extent of hepatic lipid peroxidation measured by TBARS and free MDA concentration was significantly higher in the vitamin E-deficient animals than in the controls. A greater difference between the two groups was observed in the free MDA levels. ALDH activity did not respond to the increased lipid peroxidation resulting from long-term vitamin E deficiency. The activity of the enzyme seemed slightly suppressed in vitamin E-deficient tissues as compared to vitamin E-supplemented tissues, although the difference was not statistically significant. The results suggest that hepatic cytosolic ALDH is not induced by increased substrate MDA and may not be actively involved in the metabolism of MDA in liver tissues in the chronic state of vitamin E deficiency. Accordingly, high levels of TBARS and free MDA in the vitamin E-deficient liver tissues seem to be the consequence of increased membrane lipid peroxidation and decreased rate of MDA metabolism.

A severe depletion of α-tocopherol in hepatic tissue was seen in the vitamin E-deficient group, whereas the control

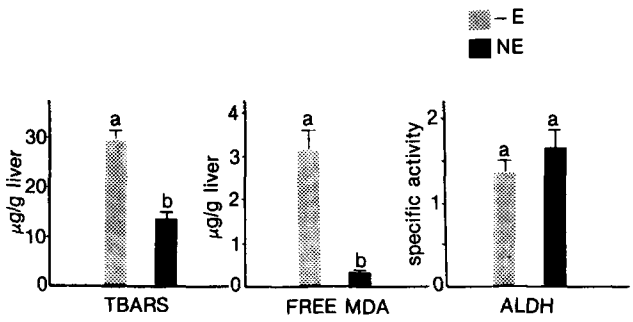

FIG. 1. The levels of thiobarbituric acid reactive substance (TBARS), free malondialdehyde (MDA) and cytosolic aldehyde dehydrogenase (ALDH) activities of liver tissues from rats fed vitamin E-deficient (–E) or vitamin E-supplemented (NE) diets for 38 wk. The levels of TBARS are represented as MDA equivalents. Enzyme activities are expressed as nmol of NADH produced per min per mg protein. Values represent means ± SEM of ten samples per treatment. Different letters on the top of the bars indicate significant differences between two groups ($P < 0.05$).

group showed hepatic α-tocopherol concentration of 35 μg/g tissue (Table 3). Plasma TBARS levels were measured to quantitate lipid peroxidation products present in the circulatory system. The level of plasma TBARS (Table 3) was significantly higher in the vitamin E-deficient condition as compared to the normal condition. The magnitude of this difference was as great as that observed in the liver tissues. Such increased plasma TBARS may be due to peroxidation of erythrocyte membrane lipids and other blood lipids or the mobilization of peroxidation products from other tissues as well. There are two possible ways whereby TBARS in the circulatory system could be eliminated. One is diffusion into the hepatic tissue to be metabolized, because liver is the major site of aldehyde oxidation and detoxification (22). The other way is excretion through urine.

Effect of dietary PUFA on in vivo *lipid peroxidation.* The TBARS, free MDA production and ALDH activity in the liver of rats fed different levels of dietary PUFA for 16 wk are shown in Figure 2. The levels of hepatic TBARS and free MDA were significantly higher in the HP group than in the LP and MP groups. This result is in agreement with the work done byHafeman and Hoekstra (23), who demonstrated that the substitution of dietary cod liver oil for dietary lard caused a large increase in ethane evolution.

TABLE 3

Effect of Dietary Vitamin E on the Levels of Plasma TBARS and Liver α-Tocopherol of Rats

Group	α-Tocopherol (μg/g liver)	Plasma TBARS[a] (ng/mL plasma)
–E	1.43 ± 0.21[b]	125.96 ± 15.63[b]
NE	34.74 ± 3.06[c]	34.84 ± 4.30[c]

[a]TBARS, thiobarbituric acid reactive substances; –E, vitamin E-deficient diet; NE, diet supplemented with 30 ppm vitamin E.
[b,c]Values within each column not sharing a common superscript letter are significantly different ($P < 0.05$) by Student's *t*-test. Means ± SEM for ten rats per treatment.

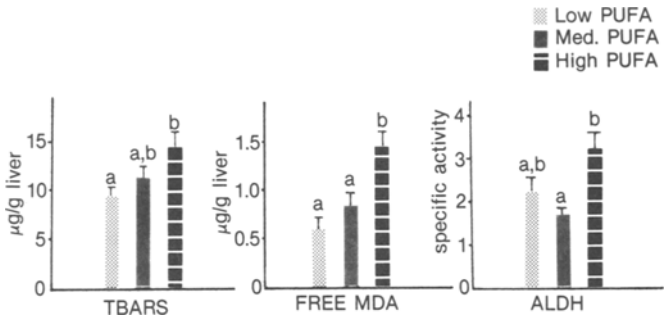

FIG. 2. The levels of TBARS, free MDA and cytosolic ALDH activities of liver tissues from rats fed diets with different levels of polyunsaturated fatty acid (PUFA) for 16 wk. The levels of TBARS are represented as MDA equivalents. Enzyme activities are expressed as nmol of NADH produced per min per mg protein. Values represent means ± SEM of ten samples per group. Different letters on the top of the bars indicate significant differences between groups by a multiple comparison test ($P < 0.05$). Abbreviations as in Figure 1.

Cod liver oil, which comprised 5% of the HP diet in this study, is a rich source of n-3 unsaturated fatty acids. Consumption of cod liver oil by animals is known to increase the levels of these fatty acids in both adipose fat and membrane phospholipids (24). Draper *et al.* (25) confirmed the enrichment of liver tissue and epididymal fat pads with eicosapentaenoic (20:5n-3) and docosahexaenoic acid (22:6n-3) in rats fed an HP diet containing cod liver oil. Therefore, lipid peroxidation in tissues of rats fed an HP diet containing cod liver oil is expected to increase because these highly unsaturated n-3 fatty acids are good substrates for lipid peroxidation.

The activity of hepatic ALDH was significantly increased in the HP group as compared to the LP group. However, no significant difference was observed between the LP and MP groups. In the present experiment, hepatic ALDH appears to respond to the increased tissue lipid peroxidation and may be involved in the metabolism of MDA in the animals fed an HP diet with normal levels of vitamin E supplementation. However, this finding is in contrast to previous result showing suppressed ALDH activity in spite of increased tissue lipid peroxidation in long-term vitamin E deficiency.

Tocopherol concentrations in liver tissues of rats fed different levels of dietary PUFA are shown in Table 4. Overall, tissue tocopherol levels were 30% lower than the value (35 μg/g tissue) found in a group fed the same amount of dietary vitamin E and 8% corn oil (Experiment 1) (Table 3). This difference must be due to the increased consumption of dietary fat (15%) by the animals. However, the HP group had 20% less hepatic vitamin E storage than the other two groups, but the difference was not statistically significant in the LP, MP and HP groups.

Plasma TBARS levels also showed a positive correlation with the amounts of dietary PUFA (Table 4). The differences in plasma TBARS levels among the dietary groups showed the same pattern as those in hepatic TBARS levels.

Effects of dietary Se and vitamin E on in vivo *lipid peroxidation.* The effects of Se and vitamin E on liver TBARS,

TABLE 4

Effect of Different Levels of Dietary PUFA on the Levels of Plasma TBARS and Liver α-Tocopherol of Rats[a]

Group	α-Tocopherol (μg/g liver)	Plasma TBARS (ng/mL plasma)
LP	24.95 ± 2.14[b]	75.52 ± 2.21[b]
MP	24.24 ± 2.30[b]	81.60 ± 3.72[b]
HP	19.55 ± 1.94[b]	107.00 ± 4.68[c]

[a]Abbreviations as in Tables 1 and 3; LP, low-PUFA diet; MP, medium-PUFA diet; HP, high-PUFA diet.
[b,c]Values within each column not sharing a common superscript letter are significantly different ($P < 0.05$) by Tukey's multiple comparison test. Means ± SEM for ten rats per treatment.

free MDA levels and ALDH activity are shown in Figure 3. The doubly-deficient group produced the highest amounts of TBARS and free MDA in liver tissues, followed by the vitamin E-deficient group. Notably, the production of free MDA in liver tissues of these two groups was significantly higher than that of the control group. The increase in tissue lipid peroxidation caused by Se deficiency alone was not statistically significant, nor was an apparent additive effect of vitamin E and Se on the induction of *in vivo* lipid peroxidation seen in this experiment. It has been generally assumed that if Se deficiency were to exist, *in vivo* peroxidation would be increased because of an inability of the cells to scavenge H_2O_2 and a decreased ability to handle lipid hydroperoxides. However, the influence of Se deficiency on the extent of lipid peroxidation in isolated organs or in the intact animal is not yet conclusive. Although a vital role of Se was established for the seleno-enzyme, glutathione peroxidase, in the reduction of hydroperoxides *in vitro* (26), significant lipid

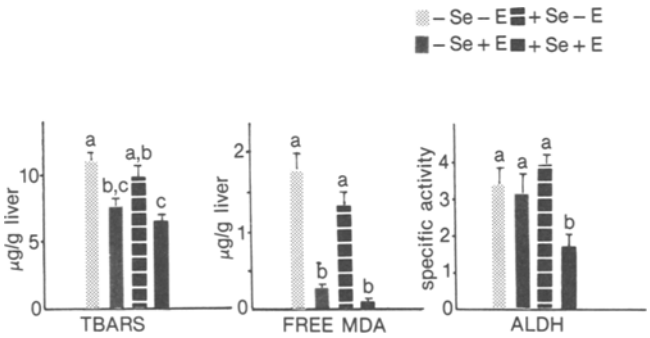

FIG. 3. The levels of TBARS, free MDA and cytosolic ALDH activities of liver tissues from rats fed diets deficient in or supplemented with selenium or vitamin E for 12 wk. The levels of TBARS are represented as MDA equivalents. Enzyme activities are expressed as nmol of NADH produced per min per mg protein. Values represent mean ± SEM of tensamples per treatment. Different letters on the top of the bars indicate significant differences between groups by multiple comparison test ($P < 0.05$). −Se−E, selenium- and vitamin E-deficient diet; −Se+E, diet supplemented with 30 ppm vitamin E as *RRR*-α-tocopheryl acetate; +Se−E, diet supplemented with 0.2 ppm selenium as sodium selenite for the vitamin E-deficient group; +Se+E, diet supplemented with 30 ppm vitamin E as *RRR*-α-tocopheryl acetate and 0.2 ppm selenium. Other abbreviations in Figure 1.

peroxidation could not be detected in Se-deficient, vitamin E-supplemented rats as measured by the alkane production in exhaled air (23). In contrast, Doni *et al.* (27) reported that after a three-month feeding, heart and kidney MDA, as measured by the TBA tests, were significantly higher both in vitamin E-deficient and Se-deficient rats than in control rats. It was found in the present study that *in vivo* lipid peroxidation is more dependent on vitamin E deficiency than on the Se deficiency. This is in agreement with the results of Hafeman and Hoekstra (28), who reported that vitamin E had a greater effect than Se in reducing ethane evolution after CCl_4 administration to rats, suggesting that vitamin E is a more potent biological antioxidant than Se. The failure of MDA to accumulate in Se deficiency may be explained by the fact that the primary substrate for Se-dependent glutathione peroxidase is H_2O_2 rather than fatty acyl peroxides (29). A further explanation may be that Se-independent glutathione peroxidase is present in tissues (30).

Hepatic ALDH activity increased significantly in the Se- or vitamin E-deficient groups, as well as in the double-deficient group compared to the control group. In the vitamin E and doubly-deficient groups, the enzyme activity appears to increase in response to the increased hepatic lipid peroxidation; however, ALDH is activated in Se deficiency in spite of low lipid peroxidation in liver tissue. Such an increase in the activity of ALDH may be explained as a compensatory action of the enzyme for the decreased glutathione peroxidase activity usually observed in Se deficiency (31). Other antioxidant enzymes, such as catalase and superoxide dismutase, were also reported to increase their activities in liver and brain of chicks during Se deficiency (32).

The α-tocopherol content of liver tissue showed a correlation with dietary tocopherol levels (Table 5). The control and Se-deficient groups had significantly higher storage levels of liver α-tocopherol than the vitamin E-deficient and the doubly-deficient groups. The decrease in tissue α-tocopherol level produced by Se deficiency was not statistically significant. Observations by several investigators have indicated that Se deficiency can affect α-tocopherol metabolism. Fisher and Whanger (33) showed a greater rate of tritium excretion in the urine of Se-deficient rats than that of controls after administration of tritiated α-tocopherol. They concluded that vitamin E is metabolized more rapidly in Se-deficient rats than in controls. This finding is consistent with the report that Se deficiency in rats depressed hepatic microsomal α-tocopherol content (34). Thus, Se deficiency appears to favor an increased rate of loss of a α-tocopherol from the body. The cause of this loss most likely relates to increased α-tocopherol oxidation under conditions where Se-dependent antioxidant mechanisms are impaired.

Plasma TBARS levels showed the same trend as liver tissue TBARS levels (Table 5), demonstrating that plasma TBARS levels can be as useful an index of *in vivo* lipid peroxidation as are liver tissue TBARS levels.

The extent of hepatic lipid peroxidation was determined by measuring the accumulation of free MDA and TBARS in the presence or absence of dietary vitamin E and/or Se and at various levels of PUFA administration. The response of hepatic cytosolic ALDH to the tissue lipid peroxidation state was examined. Hepatic lipid peroxidation was significantly increased by vitamin E deficiency and by high levels of PUFA intake. The increase of hepatic lipid peroxidation by Se deficiency was not statistically significant. The hepatic cytosolic ALDH responded to the increased tissue lipid peroxidation after 12 or 16 wk of feeding diets deficient in vitamin E, diets deficient in both vitamin E and Se, or diets with high levels of PUFA. However, hepatic ALDH activity was found to be suppressed after 38 wk of feeding diets deficient in vitamin E. These results indicate that liver cytosolic ALDH may be involved in the metabolism of MDA during *in vivo* lipid peroxidation induced by relatively short-term feeding, but the enzyme activation does not persist during severe *in vivo* lipid peroxidation caused by long-term vitamin E deficiency.

Liver α-tocopherol levels showed an inverse relationship with the extent of tissue lipid peroxidation. However, the decreases in tissue α-tocopherol levels at high levels of PUFA administration and Se deficiency were not significant. Levels of plasma TBARS showed exactly the same patterns as those of the hepatic tissues, indicating that the measurement of plasma TBARS can be used as an index for *in vivo* lipid peroxidation.

TABLE 5

Effect of Dietary Se and Vitamin E on the Levels of Plasma TBARS and Liver α-Tocopherol of Rats

Group[a]	α-Tocopherol (μg/g liver)	Plasma TBARS (ng/mL plasma)
–Se–E	1.96 ± 0.06[b]	75.80 ± 4.32[b]
–Se+E	32.48 ± 1.99[c]	55.27 ± 2.95[c]
+Se–E	2.07 ± 0.05[b]	74.81 ± 3.36[b]
+Se+E	34.18 ± 2.47[c]	52.08 ± 4.91[c]

[a]-Se-E, selenium- and vitamin E-deficient diet; -Se+E, diet supplemented with 30 ppm vitamin E as RRR-α-tocopheryl acetate; +Se-E, diet supplemented with 0.2 ppm selenium as sodium selenite for the vitamin E-deficient group; +Se+E, diet supplemented with 30 ppm vitamin E as RRR-α-tocopheryl acetate and 0.2 ppm selenium. Other abbreviation in Table 3.

[b,c]Values within each column not sharing a common superscript letter are significantly different ($P < 0.05$) by Tukey's multiple comparison test. Means ± SEM for ten rats per treatment.

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Effects of Dietary Oils and Methyl Ethyl Ketone Peroxide on *in vivo* Lipid Peroxidation and Antioxidants in Rat Heart and Liver

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Weanling male Sprague-Dawley rats were fed diets for four weeks which differed in their content of n-6 (corn oil; CO) and n-3 fatty acids (fish oil; FO), but were similar in their content of saturated and monounsaturated fatty acids and vitamin E. At the end of the four-week feeding period, each dietary group was subdivided into two groups. One group received a single placebo injection of α-tocopherol-stripped corn oil (TSCO); the other group received a single injection of the free radical generator, methyl ethyl ketone peroxide (MEKP), in TSCO. Twenty-four hours after injection, the effect of dietary oil and MEKP treatment on endogenous lipid peroxide (LPO) production (measured as methylene blue formed by the "Determiner LPO" assay), glutathione (GSH) and vitamin E content, and fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in heart and liver from unfasted animals were measured. FO-fed rats had significantly heavier hearts and livers, increased levels of n-3 fatty acids in membrane phospholipids, and higher liver LPO levels than CO-fed rats. MEKP treatment resulted in significantly lower body weights and liver GSH levels. The data indicate that dietary n-3 fatty acids increase lipid peroxidation in liver somewhat more than in heart. The study also demonstrates that the effect of induced oxidative stress due to a single dose of MEKP on lipid peroxide formation and antioxidant status in tissues from unfasted animals was independent of the dietary oils.

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Dietary supplementation with highly polyunsaturated fats, such as those containing the n-3 fatty acids found in fish oil, is associated with an increased tendency for lipid peroxide formation in certain tissues (1–3). Lipid peroxidation mediated by free radicals is considered to be a prevalent mechanism of cell membrane destruction and cell damage and has been suggested to be associated with the initiation and development of atherosclerosis (4). Little is known about how the organism responds to an increase in oxidative stress (i.e., activation of free-radical reactions), such as lipid peroxidation. In view of the suspected role of oxidative degradation of lipids in the etiology of atherogenesis, such information is useful.

Dietary fatty acids influence the composition of membrane phospholipids in heart and liver (5,6). Indeed, membrane phospholipids with a high level of polyunsaturated fatty acids (PUFA) are more sensitive to hydroxy radicals (7,8). Susceptibility to peroxidation is also influenced by free radical scavengers and antioxidants such as vitamin E and glutathione (GSH) (9). Induced oxidative stress is accompanied by a decrease in the content of the antioxidants vitamin E and/or GSH (10). Further, increased lipid peroxidation is associated with a decrease in liver GSH (11–13).

In the above experiments, animals were fasted overnight prior to sampling. The standard practice to fast animals overnight before drug administration in experimental research is to ensure uniform absorption of the test compound. However, deprivation of food and/or water is a powerful stressor. Experiments in rats have shown that food deprivation induces alterations in free radical-scavenging systems that differ from tissue to tissue (14). Elevated turnover rates of liver GSH have been reported during fasting in rats (15). Furthermore, fasting in rats lowers the GSH level of the liver without changing its level in the heart (15–17).

Data that indicate that feeding fish oil (FO) induces higher lipid peroxidation in tissues has been based on the thiobarbituric acid reactive substances (TBARS) test. This method measures the end product, malondialdehyde, which results from the breakdown of lipid peroxides (LPOs) and nonlipid compounds. Several investigators have criticized the use of the TBARS test since the test is not a reliable index of lipid peroxidation in organ systems (18–20). A preferable procedure is to measure LPO levels, using the Determiner LPO assay, a colorimetric method that specifically detects hydroperoxides, endoperoxides and peroxy radicals (20,21).

The present study was undertaken to investigate the effects of dietary n-6 and n-3 PUFA and free radicals generated by methyl ethyl ketone peroxide (MEKP) on the extent of lipid peroxidation and on antioxidant status, i.e., vitamin E and GSH content, in heart and liver tissues of rats. MEKP is a toxic, lipid-soluble peroxide that has been shown to rapidly initiate lipid peroxidation *in vivo* and *in vitro* (22,23). To avoid the possible potentiating effect of fasting on detoxification systems, the effect of MEKP on *in vivo* oxidation processes was studied in unfasted normal animals. The lipid peroxidation was measured by a new method, the Determiner LPO assay (20,21).

MATERIALS AND METHODS

Animals and diets. Forty-one male weanling Sprague-Dawley rats were obtained from Simonson Laboratories (Los Angeles, CA). The rats were housed individually in wire cages under standard conditions of 22°C, 45% relative humidity, using a 12-h light–dark cycle. The animal care procedures used in this study were approved by the Animal Care and Use Committee at Oregon State

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Abbreviations: CO, corn oil; FO, fish oil; GSH, glutathione; GSSH, oxidized GSH; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; LPO, lipid peroxide; LSD, least significant difference; MEKP, methyl ethyl ketone peroxide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, peroxidizability index; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroquinone; TSCO, α-tocopherol stripped corn oil.

University (Corvallis, OR). Following a one-week acclimation period, the rats were randomized into two dietary groups and maintained for four weeks on diets that differed in their polyunsaturated n-6 (corn oil diet; CO diet) and n-3 (FO diet) fatty acid contents. The compositions of the diets were based on the AIN-76 diet for rats (24) with part of the carbohydrate replaced with fat so that both diets were 20% fat by weight. The base diet was a fat-free, vitamin E-deficient diet (Teklad, Madison, WI). The CO diet contained 15% vitamin E-stripped corn oil (TSCO; Teklad), 4.5% cocoa butter and 0.5% coconut oil. The FO diet contained 16.75% FO, 2.0% TSCO and 1.25% olive oil. Small amounts of hydrogenated coconut oil (Teklad), olive oil (Teklad), and/or cocoa butter (Teklad) were added to the diets to equalize the contents of monounsaturated and saturated fatty acids. In addition, the diets contained the following (g/kg): casein 200, methionine 3, sucrose 350, cornstarch 150, cellulose 50, mineral mix 35, vitamin mix 10 and choline bitartrate 2. The cholesterol content of the diets was matched at 0.34 g/kg by adjusting the CO diet. dl-α-Tocopheryl acetate (a generous gift from Eastman Kodak, Kingsport, TN) was added to the diets at a level of 50 IU per kg diet. FO was obtained from the National Institutes of Health Fish Oil Test Material Program with tertiary butylhydroquinone (TBHQ) (0.2 g/kg oil) added as an antioxidant. TBHQ was added to the CO diet in an amount equal to that of the FO diet. Twenty-four hour food intake was measured each week, and there were no significant differences observed between the two dietary groups.

The fatty acid composition (g/100 g) of the diets is shown in Table 1. To minimize oxidation, diets were prepared biweekly, aliquoted into daily portions and stored frozen under nitrogen. The diets given to the rats were changed daily, and unused portions were discarded. The rats received their respective diets and water *ad libitum* throughout the study.

At the end of the four-week feeding period, each diet group was subdivided into two groups. One group received a single intraperitoneally (i.p.) placebo injection of α-tocopherol-stripped corn oil (TSCO); the other group received (i.p.) a single dose (40 mg/kg body wt) of MEKP in TSCO. The same volume (0.7 μL/g animal) of placebo and MEKP was injected. The body weight was recorded once each week, immediately before and 24 h after TSCO or MEKP administration. The unfasted rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) 24 h after TSCO or MEKP administration. Beginning at 08:00 h every day, for five days, one animal was anesthetized each 1/2 h until 12:00 h. This was done to circumvent diurnal variation of liver GSH levels. The abdomen was opened to provide access to the vena cava. Heart and liver were thoroughly perfused with isotonic saline solution, rapidly excised, weighed, quickly frozen in liquid nitrogen and pulverized at liquid nitrogen temperature, then stored at –70°C until analyzed.

Analysis of fatty acids. Heart and liver lipids were extracted by the method of Bligh and Dyer (25). A portion of this extract was used for measuring LPOs. 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) developed in a solvent system consisting of chloroform/methanol/acetic acid/water (75:40:12:5, by vol). The phospholipid fatty acids were transmethylated for 90 min at 95°C using 14% boron trichloride/methanol (Sigma Chemical Co., St. Louis, MO). The methyl esters were analyzed using a gas chromatograph (Hewlett-Packard 5890; Hewlett-Packard, Palo Alto, CA) equipped with a capillary column, 30 m × 0.25 mm i.d., coated with a 0.25-μm film of SP2330 (Supelco, Bellefonte, PA). The oven temperature was programmed to maintain 170°C for 4 min, then was increased at 2°C/min to 200°C, and then at 3°C/min to 218°C. The carrier gas was helium at a flow rate of 0.6 mL/min. The fatty acid methyl ester peaks were calibrated against those of commercial standards (Sigma; Nu-Chek-Prep, Elysian, MN). The peroxidizability index (PI) was calculated as follows: PI = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

Quantitative analysis of LPO. LPOs were quantitatively determined by measuring colorimetrically the methylene blue formed using a Determiner LPO assay kit (Kamiya Biomedical Company, Thousand Oaks, CA), modified as discussed by Turini *et al.* (20). One-half milliliter of the first reagent (275 U/mL ascorbic oxidase, 35 U/mL lipoprotein lipase, stabilizer, 50 mM Good's buffer and surfactants) was added to 100 μL of heart or liver lipid extract in methanol, which contained approximately 10 mg protein. Tubes were mixed and incubated in a shaking water

TABLE 1

Fatty Acid Composition (g/100 g) of the Diets[a]

Diet	Corn oil	Fish oil
Fatty acids		
14:0	0.11	1.55
16:0	2.96	3.02
16:1n-7		2.01
18:0	2.06	0.54
18:1n-9	5.47	2.69
18:1n-7	0.12	0.54
18:2n-6*t*		0.29
18:2n-6*c*	9.55	1.60
18:3n-3	0.18	0.21
18:4n-3		0.55
20:1n-9		0.19
20:4n-6		0.14
20:5n-3		2.40
22:4n-6		0.15
22:5n-3		0.36
22:6n-3		1.49
Others[b]	0.13	2.56
Total saturated	5.26	5.46
Total monoenoic	5.64	5.55
Total (n-6)	9.55	2.25
Total (n-3)	0.18	5.01

[a]Values represent the average of three determinations. Dietary fat was 20% by weight of the diet.

[b]Minor components (percent at less than 1% when expressed as weight percent of the total fatty acids).

bath at 30°C for 5 min. One milliliter of the second reagent (2.2 μmol MCDP, i.e., 10-*N*-methyl-carbamoyl-3,7-dimethylamino-10H-phenothiazine, plus 50 mM Good's buffer, 33.8 μg/mL hemoglobin and surfactants) was added to the tubes. The contents were then mixed and incubated at 30°C for 10 min. The absorbance was measured at 675 nm in a Gilford Model 252 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH). Sample concentrations were calculated using a standard solution of 50 nmol/mL cumene hydroperoxide. LPO values are expressed as nmole equivalents of cumene hydroperoxides per mg tissue protein. Protein was measured by the method of Lowry *et al.* (26) on the heart and liver homogenates.

Quantitative analysis of GSH and vitamin E. The procedures for the measurement of reduced GSH and for the extraction and analysis of vitamin E from heart and liver were those by Warren and Reed (10). Care was taken to avoid substantial exposure of samples to light and air. Briefly, an internal standard of δ-tocopherol and water were added to a portion (approximately 300 mg) of frozen, pulverized heart or liver tissue that was then thoroughly mixed by sonication on ice. Portions of the heart and liver homogenates were removed for measurement of protein, GSH and vitamin E. GSH levels were measured by high-performance liquid chromatography (HPLC) (27). Vitamin E was extracted with heptane and quantitated by reverse-phase HPLC using fluorimetric detection (10,28). Values for GSH and vitamin E are expressed as nmol/mg tissue protein.

Statistical analysis. The effect of dietary oil and treatment was examined by two-way analysis of variance using SAS (29) general linear model procedures. Where significant interactions were found, comparisons of different treatments in rats receiving either FO- or CO-enriched diet were performed with the Fisher's least significant difference (LSD) test (30). Differences were considered significant at $P < 0.05$.

RESULTS

The weights of the animals at the end of the feeding trial before the injection of TSCO or MEKP were not significantly different (Table 2). By 24 h, injection of MEKP decreased the weight of rats in both dietary groups ($P < 0.01$). Figure 1 shows the change in body weight 24 h after TSCO or MEKP treatment, compared to the average weight gain per 24 h over the four-week feeding period in both dietary groups. There was no significant difference in body weight gains per 24 h between the four groups over the four-week feeding period. However, a single injection of TSCO led to a slightly smaller increase in weight in the 24 h interval following the injection compared to the gain previously experienced in both dietary groups (4.6 ± 1.2 g in CO-fed group, 3.5 ± 1.8 g in FO-fed group vs. 6.4 ± 0.2 g and 6.5 ± 0.03 g, respectively). A single injection of MEKP caused a body weight loss within 24 h in both dietary groups that was more pronounced in the CO-fed rats (–6.0 ± 1.7 g in CO-fed group and –2.2 ± 1.4 g in FO-fed group).

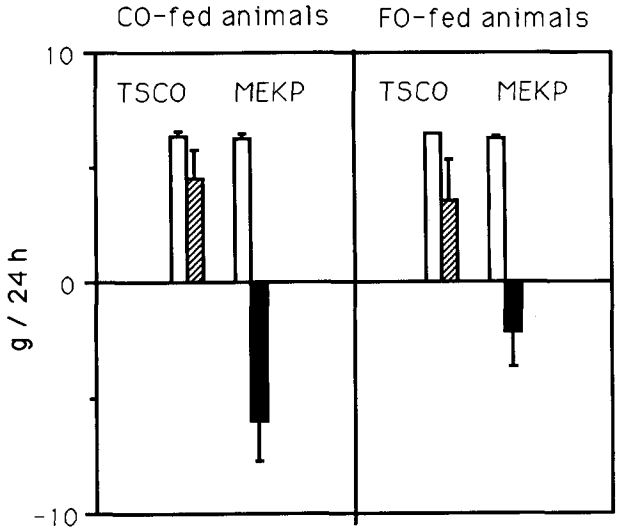

FIG. 1. Average weight gain (g) per 24 h over the four-week feeding period (open bars); weight gain (g) within 24 h following TSCO (tocopherol-stripped corn oil) treatment (hatched bars); and weight loss (g) within 24 h following MEKP (methyl ethyl ketone peroxide) treatment (full bars). CO, corn oil; FO, fish oil.

Body, heart and liver weights of rats fed CO- or FO-rich diets and treated either with TSCO or MEKP are shown in Table 2. The two diets led to significant differences ($P < 0.05$) in the mean and relative mean weights of hearts and livers. Rats fed the FO-rich diet had significantly

TABLE 2

Effect of Dietary Oil and MEKP Treatment on Body, Heart and Liver Weights[a]

	Corn oil		Fish oil		*P*-value[b]		
	TSCO	MEKP	TSCO	MEKP	O	T	O × T
Body weight at injection (g)	255.5 ± 5.4	250.7 ± 6.2	261.6 ± 5.4	251.1 ± 5.5	NS	NS	NS
Body weight at kill (g)	260.1 ± 5.7	244.7 ± 6.6	265.1 ± 5.5	248.9 ± 5.4	NS	0.01	NS
Heart weight (g)	1.08 ± 0.03	1.03 ± 0.04	1.16 ± 0.03	1.12 ± 0.03	0.02	NS	NS
Relative heart weight (%)[c]	0.41 ± 0.01	0.42 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	0.03	NS	NS
Liver weight (g)	11.92 ± 0.48	11.20 ± 0.36	14.11 ± 0.54	11.80 ± 0.69	0.01	0.007	NS
Relative liver weight (%)[c]	4.59 ± 0.19	4.59 ± 0.15	5.35 ± 0.25	4.74 ± 0.26	0.04	NS	NS

[a]Values are mean ± SEM for 10–11 rats. TSCO, α-tocopherol-stripped corn oil; MEKP, methyl ethyl ketone peroxide.

[b]NS, not significant at $P < 0.05$; O, effect of dietary oil; T, effect of MEKP treatment.

[c]Expressed as percent, g tissue per g body weight × 100.

heavier hearts and livers than those fed the CO-rich diet. These differences prevailed when the data were expressed on a relative weight basis. The livers of the MEKP-treated rats were lighter than those that did not receive the injection, when the data were expressed on a absolute basis but not on a relative basis.

The highly polyunsaturated n-6 and n-3 fatty acids in the diets were efficiently incorporated into the major classes of membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE; data not shown), of heart and liver. The fatty acid levels of phospholipids in heart and liver were significantly different between the two dietary groups with respect to most of the fatty acids analyzed. Pronounced increases in n-3 fatty acids in heart and liver membrane phospholipids have been shown repeatedly, and since the effect of the treatment and the interaction between the effects of dietary oil and treatment caused minimal changes in the fatty acid levels of heart and liver PE, the data from PE are not given here. The interaction between dietary oil and treatment was significant in six fatty acids and in the PI of heart PC (Table 3) and in two fatty acids of liver PC (Table 4). Within the CO group, MEKP treatment significantly decreased the level of 18:2n-6. Within the FO group, the level of 16:0 was significantly increased, and the levels of 20:4n-6, 22:5n-3, 22:6n-3 and the PI were significantly decreased after a single MEKP injection. MEKP treatment changed the fatty acid levels of liver PC only within the CO group, where the levels of 18:1n-9 and 18:2n-6 were decreased. In heart PE, there was one interaction where the PI values decreased in MEKP-injected FO-fed rats from 239.2 ± 3.3% to 221.3 ± 4.3% but remained virtually unchanged in CO-fed rats (148.8 ± 2.0% to 146.6 ± 4.5%).

The effects of the dietary oils and MEKP treatment on GSH, vitamin E and LPO levels are shown in Table 5. Liver GSH was significantly reduced ($P < 0.003$) by MEKP treatment while dietary oils had no effect. Heart GSH was unaffected by dietary oil or MEKP treatment. Liver GSH was roughly three times that of the heart. Vitamin E in heart and liver was unaffected by dietary oil or MEKP treatment. However, heart vitamin E was roughly twice that in the liver (2 nmol/mg protein vs. 1, respectively). Liver LPO was significantly higher ($P < 0.05$) in the FO-fed group compared to the CO-fed group, while heart LPO was unaffected. MEKP treatment did not affect the LPO levels in heart or liver in either of the dietary groups.

Figure 2 shows the relationship between PI in heart PC, vitamin E and LPO levels from rats on the FO-diet and being MEKP-treated. The PI was positively correlated with vitamin E levels and negatively correlated with LPO levels.

DISCUSSION

The present study has shown that rats fed n-3 enriched diets do not respond differently to induced oxidative stress from a single dose of MEKP as evidenced by LPO formation and antioxidant status, compared to rats fed n-6 enriched diets. As anticipated, the injection of MEKP stressed the animals, as evidenced by their weight loss within 24 h after injection (Fig. 1). The changes in body weight effected by MEKP might be due to loss of appetite and lower food intake in addition to metabolic changes. The injection of TSCO or the handling associated with the injection was also a stressor, as evidenced by the smaller weight gain experienced by the rats after this injection.

TABLE 3

Effect of Dietary Oil and MEKP Treatment on the Fatty Acid Composition of Heart Phosphatidylcholine[a]

	Corn oil		Fish oil		*P*-value[b]		
	TSCO	MEKP	TSCO	MEKP	O	T	O × T
Fatty acids							
14:0	0.11 ± 0.11	0.06 ± 0.06	0.22 ± 0.14	0.41 ± 0.32	NS	NS	NS
16:0	14.64 ± 0.52	13.65 ± 0.50	16.36 ± 0.33	20.29 ± 2.12[d]	0.002	NS	0.05
16:1n-7							
18:0	30.26 ± 0.44	31.73 ± 0.86	27.42 ± 0.45	28.21 ± 2.45	0.03	NS	NS
18:1n-9	4.43 ± 0.16	3.94 ± 0.10	2.64 ± 0.11	3.14 ± 0.26	<0.0001	NS	0.01
18:1n-7	2.51 ± 0.17	2.42 ± 0.08	3.02 ± 0.14	3.47 ± 0.51	0.01	NS	NS
18:2n-6	13.24 ± 0.59	9.87 ± 1.01[e]	2.64 ± 0.13	2.62 ± 0.25	<0.0001	0.01	0.01
20:4n-6	28.04 ± 1.05	30.08 ± 1.30	21.35 ± 0.34	17.81 ± 1.44[d]	<0.0001	NS	0.02
20:5n-3			4.16 ± 0.25	3.43 ± 0.20		0.02	
22:4n-6	1.52 ± 0.21	0.87 ± 0.55				NS	
22:5n-3	1.04 ± 0.10	1.37 ± 0.39	3.65 ± 0.13	2.87 ± 0.28[d]	0.0001	NS	0.04
22:6n-3	2.31 ± 0.15	3.28 ± 0.60	17.48 ± 0.20	13.66 ± 1.68[e]	0.0001	NS	0.02
n-6/n-3	12.83 ± 1.19	10.23 ± 1.79	0.95 ± 0.01	1.05 ± 0.11	0.0001	NS	NS
PI[c]	118.21 ± 2.93	124.75 ± 6.73	184.88 ± 2.09	149.58 ± 13.31[e]	0.0001	NS	0.02

[a]Data are expressed as relative weight percent of total fatty acids. Values are means ± SEM for five rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$, analysis of variance.

[c]PI, peroxidizability index, (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

[d]$P < 0.05$, [e]$P < 0.01$, compared to TSCO-treated rats, Fischer's least significant difference.

TABLE 4

Effect of Dietary Oil and MEKP Treatment on the Fatty Acid Composition of Liver Phosphatidylcholine[a]

	Corn oil		Fish oil		*P*-value[b]		
	TSCO	MEKP	TSCO	MEKP	O	T	O × T
Fatty acids							
14:0	0.35 ± 0.09	0.30 ± 0.08	0.86 ± 0.08	0.85 ± 0.11	0.0001	NS	NS
16:0	19.33 ± 0.60	21.13 ± 1.24	28.26 ± 0.88	30.08 ± 2.05	0.0001	NS	NS
16:1n-7			2.24 ± 0.16	1.70 ± 0.45		NS	
18:0	22.04 ± 0.60	24.10 ± 0.37	14.08 ± 0.30	14.54 ± 0.40	0.0001	0.010	NS
18:1n-9	4.73 ± 0.07	3.65 ± 0.22[d]	6.14 ± 0.28	6.40 ± 0.44	0.0001	NS	0.03
18:1n-7	1.33 ± 0.14	1.18 ± 0.09	2.60 ± 0.15	2.73 ± 0.16	0.0001	NS	NS
18:2n-6	16.14 ± 0.29	13.99 ± 0.30[e]	7.95 ± 0.22	8.51 ± 0.41	0.0001	0.02	0.001
20:4n-6	27.70 ± 0.46	27.71 ± 0.64	11.25 ± 0.98	9.93 ± 0.60	0.0001	NS	NS
20:5n-3			10.29 ± 1.17	9.08 ± 1.06		NS	
22:4n-6	0.65 ± 0.18	0.54 ± 0.22				NS	
22:5n-3	0.46 ± 0.23	0.67 ± 0.07	1.93 ± 0.18	1.70 ± 0.20	0.0001	NS	NS
22:6n-3	3.17 ± 0.19	3.61 ± 0.26	11.98 ± 0.45	10.87 ± 1.27	0.0001	NS	NS
n-6/n-3	12.92 ± 0.94	10.26 ± 0.81	0.87 ± 0.11	0.91 ± 0.08	0.0001	NS	NS
PI[c]	121.19 ± 1.13	120.38 ± 2.46	152.60 ± 4.07	136.99 ± 12.03	0.002	NS	NS

[a]Data are expressed as relative weight percent of total fatty acids. Values are means ± SEM for five rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$, analysis of variance.

[c]PI, peroxidizability index, (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

[d]$P < 0.05$, [e]$P < 0.001$, compared to TSCO-treated rats, Fischer's least significant difference.

This occurred despite the fact that the rats were handled frequently and that great care was taken to assure that the injection was made causing a minimum of discomfort. The data suggest that stress can confound results from studies in which animals are subjected to procedures such as an injection.

The highly polyunsaturated n-6 fatty acids in CO and n-3 fatty acids in FO were efficiently incorporated into the major classes of phospholipids, i.e., PC and PE, of heart and liver, which is in agreement with previous studies (5,6). The contribution of individual fatty acids to lipid peroxidation has been shown to reflect the degree of unsaturation of n-3 fatty acids more than that of the n-6 type (31). In our study, a single MEKP injection caused significant reduction in the levels of PUFA, 20:4n-6, 22:5n-3 and 22:6n-3 of heart PC from FO-fed rats. The interaction between dietary FO and MEKP treatment on fatty acid levels was evident to a lesser extent in heart PE (data not shown) and liver PC. The fatty acid levels of liver PE (data not shown) were unaffected by MEKP in each of the dietary groups. In sarcolemma of myocardial cells, PC is preferentially present in the outer monolayer, and PE in the inner (32). The observed reduction in the levels of highly PUFA in heart PC could indicate that PC is less

TABLE 5

Effect of Dietary Oil and MEKP Treatment on Glutathione (GSH), Vitamin E and Lipid Peroxide (LPO) Content of Heart and Liver[a]

	Corn oil		Fish oil		*P*-value[b]		
	TSCO	MEKP	TSCO	MEKP	O	T	O × T
Heart							
GSH	9.6 ± 0.5	8.9 ± 0.8	9.2 ± 0.6	9.4 ± 0.6	NS	NS	NS
Vitamin E	2.09 ± 0.10	2.03 ± 0.14	1.85 ± 0.11	2.17 ± 0.15	NS	NS	NS
LPO	0.040 ± 0.006	0.029 ± 0.004	0.033 ± 0.007	0.024 ± 0.005	NS	NS	NS
Liver							
GSH	31.2 ± 2.1	19.8 ± 2.6	28.0 ± 2.1	24.5 ± 2.5	NS	0.003	NS
Vitamin E	1.15 ± 0.13	0.90 ± 0.12	0.90 ± 0.04	0.88 ± 0.07	NS	NS	NS
LPO	0.053 ± 0.008	0.043 ± 0.007	0.066 ± 0.10	0.074 ± 0.014	0.05	NS	NS

[a]Values are reported as nmol/mg protein and are means ± SEM for 10–11 rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$.

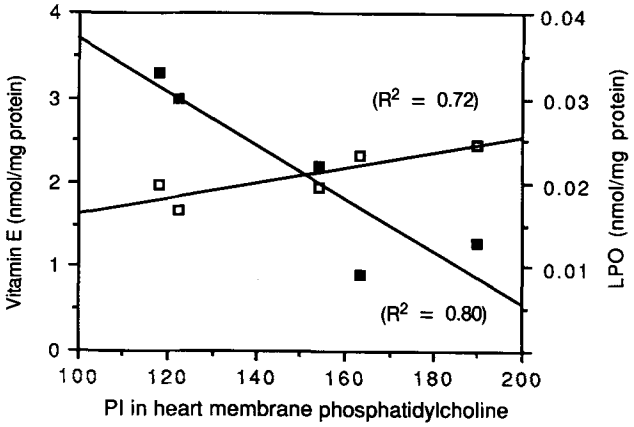

FIG. 2. Relationship between peroxidizability index (PI) in phosphatidylcholine, vitamin E (open symbols) and lipid peroxide (LPO, closed symbols) levels in heart from rats on fish oil-rich diet being methyl ethyl ketone peroxide treated.

protected from oxidation induced by MEKP. The observed changes in fatty acid composition of heart phospholipids did not resemble changes induced by stress, i.e., exogenous epinephrine (5). Repeated administration of epinephrine caused increased levels of 20:4n-6 and 22:6n-3 in PC, decreased or unaltered 20:4n-6 and increased 22:6n-3 in PE and decreased level of 18:2n-6 in PC and PE. These changes were qualitatively similar although different dietary fats were used, i.e., butter, corn oil or cod liver oil (5).

The phospholipids of heart and liver from the FO-fed rats contain high levels of the highly peroxidizable polyunsaturated n-3 fatty acids (Tables 3 and 4) causing the higher PI values. Even though the PI of the phospholipids was higher in heart than in liver of FO-fed rats, lipid peroxidation was higher in the liver, i.e., the LPO levels were higher in liver than in heart of FO-fed rats (Table 5). Vitamin E is thought to act as one of the most important antioxidants *in vivo* because of its role in blocking the propagation of LPO in the membrane. Vitamin E levels in the heart were twice those in the liver, and LPO levels were lower in heart than in liver. The insensitivity of heart from FO-fed rats to lipid peroxidation could be explained by the high vitamin E level, which seems to be sufficient to suppress the *in vivo* peroxidation of n-3 fatty acids in heart phospholipids of normal rats.

Mouri *et al.* (3) and Chautan *et al.* (33) showed that the heart vitamin E content dramatically increased as the membranes became enriched in n-3 PUFA, whereas liver became depleted of vitamin E. However, in our study, no differences in the level of vitamin E in heart or liver were found between FO-fed and CO-fed rats (Table 5). The role of vitamin E in preventing the oxidative destruction of PUFA in cell membranes has been elusive (34). It has been suggested that vitamin E stabilizes the cell membrane through interaction of its phytyl side chains with the polyunsaturated fatty acyl groups of phospholipids (35). Vitamin E levels and PUFA contents were shown to influence membrane susceptibility to lipid peroxidation, and below a certain vitamin E threshold, differences in membrane susceptibility to peroxidation could be reasonably predicted based on the content of individual peroxidizable fatty acids only (31). Furthermore, a strong positive correlation was found between heart vitamin E and n-3 fatty acids of total lipids in heart membranes of rats, which suggests that n-3 PUFA in cell membranes are efficiently protected against lipid peroxidation (33). Individual data from this study showed that only after an induced peroxidative stress, i.e., a single MEKP injection, was there a positive correlation between vitamin E levels and PI values and a negative correlation between LPO levels and PI values in hearts from FO-fed rats (Fig. 2). No such correlation was found in hearts from TSCO-treated rats in either of the dietary groups.

The products of lipid peroxidation are mainly the hydroperoxides of fatty acyl moieties. In the present study, the lower levels of highly PUFA of heart PC induced by MEKP are not reflected in higher levels of LPO in heart. It is suggested that membrane-bound phospholipase A_2 produces an "antioxidant" effect by excising LPOs from the membrane, thereby preventing them from participating in free radical propagation reactions (36). The membrane-bound phospholipase A_2 is hence considered as one of the scavenger and antioxidant enzymes that act to preserve cellular structure and function.

GSH in liver is thought to be used as a cysteine reservoir for GSH synthesis in peripheral organs during periods of enhanced consumption or limited supply (37,38) and as a defense mechanism against oxidative stress (39). It has been shown that the reduction of GSH levels in liver as a consequence of oxidative stress was more pronounced in livers from fasted than from fed rats (40). A significant reduction in liver GSH levels was only observed in unfasted rats 24 h after an MEKP injection (Table 5). The reduced level of liver GSH was not reflected in an increased oxidized level of GSH (GSSG). The liver and heart GSSG levels were less than 1 nmol/mg protein in both dietary groups (data not shown).

In summary, the effect of MEKP treatment on GSH levels on the levels of PUFA of membrane phospholipids and on tissue weights differed between heart and liver. One should therefore be cautious in extrapolating results from one tissue to another. The present results also support the idea that in studies on lipid peroxidation and radical-scavenging defense mechanisms, attention must be paid to the biochemical changes that are associated with fasting (14,41). In the present study, no deleterious effect of increased susceptibility to lipid peroxidation due to the n-3 fatty acid-enriched dietary intake was seen in heart and liver of unfasted animals. The reduced levels of highly PUFA in heart PC induced by MEKP were not reflected in higher levels of LPO in heart from FO-fed rats. The fate of the peroxidized PUFA will require further study.

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Meningioma Phospholipid Profiles Measured by ^{31}P Nuclear Magnetic Resonance Spectroscopy

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Fourteen cases of intracranial meningioma were characterized after chloroform/methanol extraction by ^{31}P nuclear magnetic resonance (NMR) spectroscopy at 202.4 MHz. Each phospholipid class detected in the extracts was identified and quantitated in terms of its molar percentage relative to the total phospholipids measured. The following phospholipids were assayed by ^{31}P NMR: phosphatidylglycerol, phosphatidic acid, diphosphatidylglycerol, ethanolamine plasmalogen, phosphatidylethanolamine (PE), lysophosphatidylinositol, phosphatidylserine, sphingomyelin, lysophosphatidylcholine (LPC), phosphatidylinositol (PI), sphingosylphosphorylcholine and phosphatidylcholine. In addition, two unidentified phospholipids were detected with resonances at 0.13 and –0.78 ppm, respectively. Three distinct types of spectra were obtained on the extracts and grouped accordingly for comparison purposes. Type 1 tumors showed unusual ^{31}P NMR profiles with low levels of PE and PI and elevated levels of LPC; type 2 tumors were characterized by low levels of the ethanolamine phospholipids and near equivalent levels of PI and LPC. The spectra of type 1 and type 2 tumors were characteristic of degenerative cells that lacked membrane permeability associated with loss of ethanolamine plasmalogen in the presence of significant phospholipid turnover. Meningiomas belonging to the third spectral type showed characteristics similar to those of normal tissues with normal levels of PE and ethanolamine plasmalogen, as well as very low levels of LPC relative to PI. Type 3 tumors lacked the characteristic signs of degeneration noted in type 1 and type 2 tumors. The data corroborate and augment *in vivo* spectroscopic findings reported earlier and demonstrate the value of ^{31}P NMR spectroscopic phospholipid analysis on lipid extracts for the characterization of meningiomas.

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Meningiomas and other types of intracranial tumors have been characterized and differentiated *in vivo* using magnetic resonance spectroscopy and magnetic resonance imaging (1–11). When ^{31}P nuclear magnetic resonance (NMR) is performed *in vivo,* spectral differences are often attributed to perturbations in phospholipid metabolism (3,7–9,11–17) as the integrated phosphomonoester and phosphodiester resonance signals, which arise from low-molecular weight, water-soluble phosphorylated phospholipid precursors, differ when comparing different tissue types.

The phosphomonoester resonance band, detected by *in vivo* methods, represents the membrane phospholipid precursors phosphocholine (PC) and phosphoethanolamine (PE), while the phosphodiester resonance band accounts for the phospholipid metabolic intermediates glycerol 3-phosphocholine and glycerol 3-phosphoethanolamine. Although it is generally true that these metabolites contribute substantially to the signal intensity of the respective resonance bands, to describe spectral changes in precursor metabolic profiles only in terms of phospholipid metabolism is often simplistic because these phospholipid precursors and products are known to play roles other than metabolic ones (18–21).

Tissue phospholipids can be extracted from surgical tissue specimens with chloroform/methanol and can be identified and quantitated by ^{31}P NMR (22). The spectroscopic phospholipid profiles thus obtained are characteristic of the disease state and can be used for classification purposes. In the present study, meningioma surgical tissue specimens were acquired from fourteen patients undergoing craniotomy and were analyzed to obtain quantitative spectral profiles. Statistical analysis of the spectral profiles permitted subclassification of the tumors into three groups thus allowing for a more refined biochemical differentiation of the disease states. Because ^{31}P NMR detects and resolves most phospholipids in the extract, novel and uncharacterized phospholipids can also be detected and quantified.

MATERIALS AND METHODS

Surgery. Human meningioma surgical tissue specimens were obtained from patients undergoing scheduled craniotomy. The unfixed tissue specimens were partitioned within five minutes into separate portions for histopathologic examination and NMR spectroscopic analysis. The portion taken for analysis was promptly submerged in liquid nitrogen for storage. Specimens ranged from 0.5 to 1.5 g. The remaining surgical tissue specimens were sectioned and examined microscopically for histologic diagnosis after staining with hematoxylin and eosin or other stains as required. Brains were also obtained from two C3H/He mice, which were euthanized by lethal injection of euthanasia-5 solution containing pentobarbital; the brains were dissected, removed and rinsed with physiologic saline to remove extraneous blood, and then were immediately

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Abbreviations: ATP, adenosine triphosphate; CPLAS, choline plasmalogen; CPLIP, choline phospholipid; DPG, diphosphatidylglycerol, cardiolipin; EDTA, (ethylenedinitrilo)tetraacetic acid; EPLAS, ethanolamine plasmalogen; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; U, unidentified phospholipid.

frozen in liquid nitrogen. The material was used to obtain reference spectra to confirm spectral assignments.

Phospholipid extraction. A simple, modified Folch extraction (23) for meningioma phospholipids was used in which the backwashing step utilized potassium salt of (ethylenedinitrilo)tetraacetic acid (EDTA), 0.2 M, in EDTA, pH 6.0 (24). Meningioma tissue specimens, frozen in liquid nitrogen, were pulverized to a fine powder using a stainless-steel mortar and pestle that had been chilled with liquid nitrogen. The tissue powder (0.5–1.5 g/tissue sample) was then added to 20 mL of chloroform/methanol (2:1, vol/vol). The homogenate, consisting of a single liquid phase, was filtered into a separatory funnel. The extract was washed with 4 mL of 0.2 M potassium EDTA and allowed to separate thoroughly for 24 h in a separatory funnel. The chloroform phase was recovered and evaporated using a rotary evaporator at 37°C.

Phospholipid solvent for ^{31}P NMR. The solvent for phospholipid analysis by ^{31}P NMR was hydrated chloroform/methanol (25,26) designed to permit the quantitative determination of phospholipids (22,24,25,27). The solvent consists of two parts: (A) Reagent-grade chloroform, containing 5% benzene-d_6 and an appropriate concentration of trimethylphosphate; and (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 Cs EDTA solution in 4 mL methanol. Benzene-d_6 is used to serve as internal field-frequency lock. The concentration of the trimethylphosphate chemical-shift and quantitation reference (7) can be varied depending on the phospholipid concentration. The Cs EDTA used is generated by titrating an aqueous suspension of EDTA with CsOH to pH 6, at which point a solution is obtained. For ^{31}P NMR analysis, a lipid sample (0.01–100 mg) is dissolved in 2 mL of Reagent A and transferred into an NMR sample tube; 1 mL Reagent B is added, and the sample is mixed thoroughly. A small amount of aqueous phase which separates is allowed to rise to the top of the sample (1 min). Ordinarily, there is no need to remove this small amount of aqueous phase, which contains no detectable amounts of phospholipids; its presence above the sample ensures that proper equilibria are maintained and that organic solvent loss during analysis is minimized.

^{31}P Magnetic resonance spectroscopy. The NMR spectrometer used was a GE 500NB system (General Electric, Fremont, CA) operating at 202.4 MHz for ^{31}P. The spectrometer was equipped with an Oxford Instruments (Oxford, United Kingdom) 500/52 magnet having an operating magnetic field of 11.75 Tesla and deuterium field-frequency locking capability. Tissue extracts were placed in standard 10-mm NMR sample tubes, and spun at 8 Hz during data acquisition. Proton broadband decoupling was used throughout. Chemical shift data are reported relative to 85% inorganic orthophosphoric acid (28); however, the primary internal reference standard was PC (chemical shift, –0.84 ppm) or trimethylphosphate. Spectrometer conditions were as follows: pulse sequence, one pulse; pulse width, 18 μs (45° flip angle); acquisition delay, 500 μs; cycling delay 500 ms; number of acquisitions, 12,000; number of points per free induction decay, 4096; acquisition time 1.02 s; sweep width ± 1000 Hz. The total average acquisition time per sample was 6 h. The free induction decay was Fourier transformed and filtered using a line broadening factor of 0.6 Hz.

Assignment of phospholipid resonances. Phospholipid signals in the ^{31}P NMR spectra were sequentially identified by adding known quantities of individual phospholipid standards (29,30). Phospholipid standards were obtained from the Sigma Chemical Company (St. Louis, MO) and Life Sciences, Inc. (Milwaukee, WI). In those instances where resonance signals were not fully resolved, spectral deconvolution was used. In the more concentrated samples, overlap between the phosphatidylserine (PS) and sphingomyelin (SM) resonances was significant enough to introduce substantial errors in the analysis. In such instances, the samples were further diluted with additional solvent mixture (25). Dilution caused a downfield chemical shift of PS while having little effect on the chemical shift of SM. This permitted resolution of the PS and SM signals and their quantitation by integration.

A spectrum of mouse brain (C3H/He) obtained using the same procedures is presented in Figure 1 to illustrate the resolving power of the method under optimal conditions. In the figure, the prominent PC resonance is off-scale so that the minor phospholipid resonances can be observed. The bottom spectrum illustrates the identification of the lysophosphatidylcholine (LPC) signal through addition of 1-stearoyl-*sn*-glycero-3-phosphocholine (0.4 mg; Sigma), which was introduced by microsyringe as chloroform solution. Alternative methods for obtaining rat brain NMR phospholipid spectra have been described (31).

Data analyses. Phospholipid concentrations in relative mole percentages were computed for all phospholipid resonances in the tumors analyzed. For each phospholipid, mean phospholipid concentrations were calculated and compared for the three spectral types using an analysis of variance. For those resonances for which significance was shown to exist (F probability, $P < 0.05$), a post-hoc Scheffé comparison procedure was applied with significance determined at the $P < 0.05$ level. Under most conditions, analysis of variance requires the assumption that the underlying variances between tested means be equal; therefore, for those resonances for which significance was established, homogeneity of variance was confirmed using Cochran's C and the Bartlett-Box F tests.

RESULTS

The fourteen meningioma spectra obtained in this study were qualitatively divided into three groups—type 1, type 2 and type 3. Representative meningioma spectra are shown in Figure 2. Starting downfield, the following phospholipids were identified: phosphatidylglycerol (PG), phosphatidic acid (PA), diphosphatidylglycerol (cardiolipin; DPG), ethanolamine plasmalogen (EPLAS), PE, lysophosphatidylinositol (LPI), PS, SM, LPC, phosphatidylinositol (PI), sphingosylphosphorylcholine (SPC) and PC. Two unidentified phospholipids, U and choline phospholipid (CPLIP), were also detected with signals at 0.13 and –0.78 ppm.

Type 1 spectra (Table 1) are characterized by low levels of the ethanolamine phospholipids and PI, and elevated

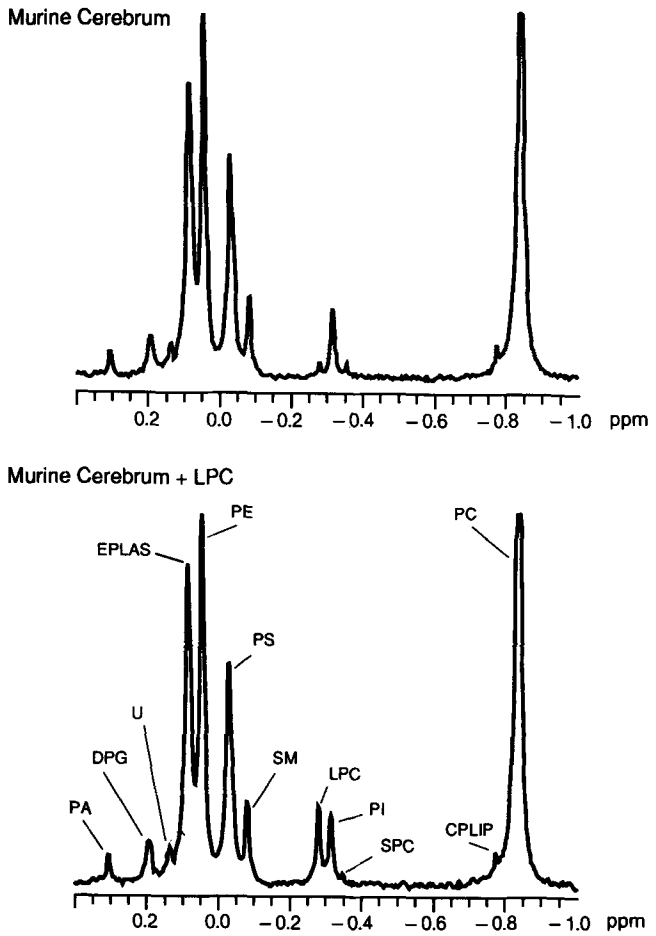

FIG. 1. ^{31}P nuclear magnetic resonance phospholipid spectrum of normal mouse cerebrum: PA, phosphatidic acid; DPG, diphosphatidylglycerol; U, an unidentified phospholipid; EPLAS, ethanolamine plasmalogen; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; SPC, sphingosylphosphorylcholine; CPLIP, choline phospholipid; and PC, phosphatidylcholine. The bottom spectrum shows the effect of adding the equivalent of 0.4 mg of LPC standard in chloroform to the sample, giving the top spectrum.

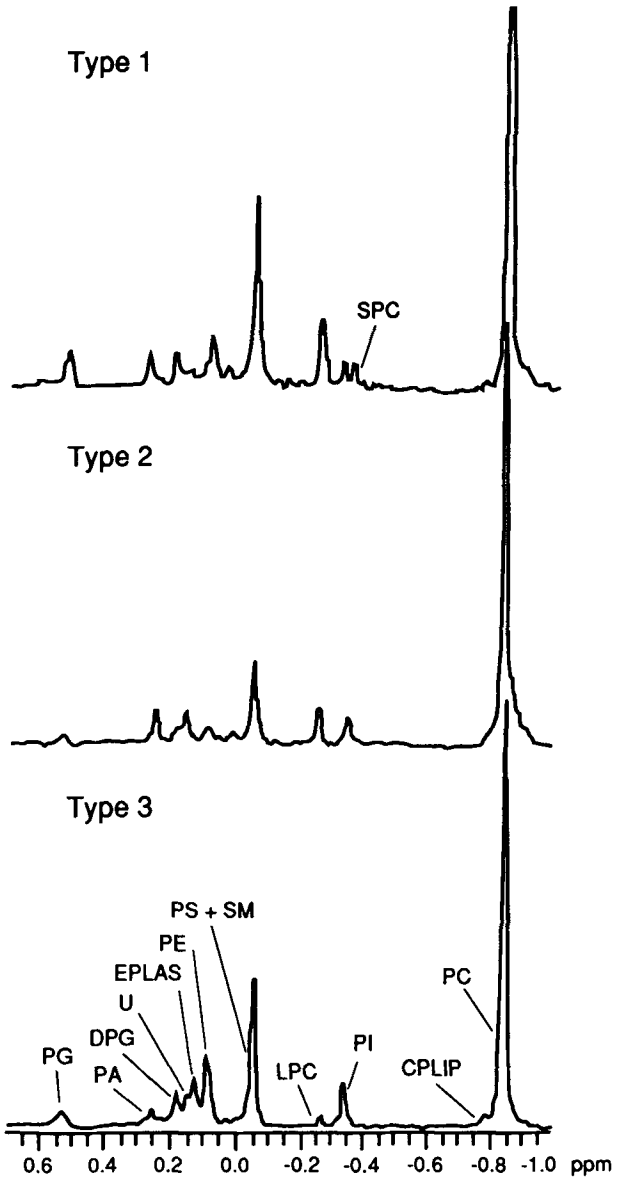

FIG. 2. Characteristic ^{31}P nuclear magnetic resonance phospholipid spectral profiles of human intracranial meningiomas. The resonance signals starting from downfield (0.6 to −1.0 ppm) are as follows: PG, phosphatidylglycerol; PA; DPG; U; EPLAS; PE; PS; SM; LPC; PI; SPC; CPLIP, choline phospholipid; and PC. Abbreviations as in Figure 1.

levels of LPC. All of these tumors were located in the vicinity of the frontal lobes, including one olfactory nerve meningioma. Three of the five tumors included in this category were considered transitional meningiomas as judged by histological analysis, while the remaining two specimens were defined as syncytial.

Type 2 spectra are unique because they contain near equivalent levels of LPC and PI and little or no ethanolamine phospholipids. Two of the three tumors were histologically syncytial, one transitional, and two out of the three were obtained from patients with recurrent disease.

Type 3 spectra include six samples that have the appearance of typical neural and nonneural tissue spectra. These tumors have very low levels of LPC relative to PI, while the presence of the ethanolamine phospholipids is noted to be within the normal range of concentrations that exists in most human tumors. This group of tumors was comprised evenly of transitional and syncytial histological types and lacked a prevalent site of origin. Temporal, frontal and parietal lobe tumors are included in this spectral type.

The relative concentration of the individual phospholipids was determined for each of the samples analyzed in this study. Subsequently, the mean relative mole percentage of the phospholipids was determined for each tissue type (Table 1). This analysis provided a basis for comparison of the three tumor types based on their phospholipid class composition. The tissue spectra all share some common features, such as the presence of PG, PA, DPG, U (0.13 ppm), PS, LPC, PI, CPLIP (–0.78 ppm) and PC. In

TABLE 1

Phospholipid Profiles of Intracranial Meningioma as Measured by ^{31}P Nuclear Magnetic Resonance[a]

Phospholipid	Relative mol% of phosphorus ± SE					
	Type 1		Type 2		Type 3	
Phosphatidylglycerol	3.63	0.42	3.84	1.39	3.40	0.46
Phosphatidic acid	4.47	0.76	4.31	0.46	2.04	0.33
Diphosphatidylglycerol	2.68	0.67	2.47	0.27	2.97	0.37
Sphingomyelin	3.42	0.55	3.04	0.50	2.58	0.32
Ethanolamine plasmalogen			2.50		9.29	1.01
Phosphatidylethanolamine	4.79	0.58	6.06	1.16	9.62	0.45
Lysophosphatidylinositol	3.18	0.32	2.30	0.005		
Phosphatidylserine	24.79	1.54	18.71	4.96	24.62	1.70
Lysophosphatidylcholine	6.80	0.68	3.49	0.84	0.82	0.09
Phosphatidylinositol	2.15	0.36	4.62	0.52	3.81	0.24
Sphingosylphosphorylcholine	2.14	0.19	1.22	0.21	0.75	0.12
Choline phospholipid	0.69	0.12	0.54	0.10	0.93	0.21
Phosphatidylcholine	41.99	0.50	48.34	6.30	39.51	1.29

[a]The profiles have been grouped into three categories based upon qualitative spectral characteristics.

TABLE 2

Statistically Significant Differences Observed Between Meningioma Phospholipid Profiles Grouped According to ^{31}P Nuclear Magnetic Resonance Spectral Characteristics

Phospholipid	Type 1 vs. type 2	Type 1 vs. type 3	Type 2 vs. type 3
Phosphatidic acid	n.s.[a]	0.05	n.s.
Phosphatidylethanolamine	n.s	0.001	n.s.
Lysophosphatidylcholine	0.01	0.001	0.05
Phosphatidylinositol	0.01	0.05	0.05
Sphingosylphosphorylcholine	0.05	0.001	0.05

[a]n.s., Not significant.

all spectra, the resonances of PC and PS dominate, whereas variations in the relative concentrations of the other phospholipids make it possible to differentiate the three meningioma groups.

The spectra of the three groups were studied using an intergroup comparison procedure (Table 1, concentration; Table 2, statistical probability value). Type 1 spectra showed significantly diminished levels of PI and increased levels of LPC and SPC when compared with type 2 spectra. In addition, the spectra showed significantly depressed levels of PI and PE, and increased levels of PA, LPC and SPC relative to type 3 spectra. Type 2 spectra showed significantly lower levels of PE and increased levels of PI, LPC and SPC when compared to type 3 spectra. LPI was absent in type 3 spectra, and EPLAS was absent in type 1 spectra and present in only 1 of 3 type 2 spectra.

DISCUSSION

In this comparison of meningioma phospholipids, a number of observations can be made: (i) based on qualitative assessment of spectral profiles, three tumor subgroup types can be identified; (ii) all three tissue types show the presence of PA and of the lysophospholipids LPI and LPC, compounds often associated with phospholipid catabolism; (iii) significant spectral differences that make it possible to distinguish between tissue types were noted for the minor phospholipids PA, LPC, PI and SPC; exceptions to this included the absence of EPLAS in type 1 and type 2 tumors and the significant differences in the levels of PE between type 1 and type 3 tumors; and (iv) significant differences between the tissue types in the level of phospholipids typically associated with the inner leaflet of the plasma membrane, such as the ethanolamine phospholipids.

The very low level of EPLAS, found in two of the three meningioma types (type 1 and type 2), is an outstanding feature of these tumors, as EPLAS can be a major component of human tissue phospholipids. For example, in the human brain myelin (27), EPLAS has been shown to account for 38% of total phospholipids, and thus exceeds even PC (24%). Most meningiomas arise from arachnoid cells and, therefore, differ from other intracranial neoplasms in their gross appearance and histology. Meningiomas are usually grossly nodular, encapsulated, and often show calcification. Histologically, the calcifications give rise to psammomatous whorls that are composed of concentrically laminated structures formed by the deposition of calcium in degenerated tumor cells. In type 3 meningiomas, the concentration of the ethanolamine phospholipids is within the expected range of other mammalian tissue types. This may reflect the profiles of those cells that are, biologically, in the early stages of the neoplastic process. Lammelar structures may be formed by degenerating cells, or by cells that have a low concentration of EPLAS. These cells are characterized by a marked decrease in membrane permeability and reflect an altered membrane messenger transport as well as poor perfusion capability. Such changes lead to a disruption of normal intracellular communication and represent end-stage processes. In meningiomas, the formation of psammoma bodies results from the precipitation of accumulated intracellular calcium or from cellular processes that allow calcium to exceed normal equilibrium concentrations. Healthy cells have a balance of ethanolamine and choline phospholipids. The absence of ethanolamine phospholipids in type 1 and type 2 tissues indicates the presence of degenerative cells that are no longer viable. Changes in phospholipid profiles may presage the degenerative processes that are often identified histologically in meningiomas. Precursors or products of these processes may serve as a means of differentiating meningiomas from other neural or non-neural tissue types. If such an interpretation were accurate, the appearance of LPI in the spectra of type 1 and type 2 meningiomas would appear to be as significant as the absence of the ethanolamine phospholipids from the same tissue types and indicative of the aforementioned degenerative processes.

According to the ^{31}P NMR measurements of Cadoux-Hudson *et al.* (3), meningiomas are more alkaline (pH = 7.19 ± 0.02) than normal brain tissue (pH = 7.03 ± 0.02). The elevated pH observed in tumor cells was ascribed to a potential alteration in Na^+/H^+ antiport activity within these cells. Similarly, Heindel *et al.* (7) reported from their studies that meningiomas were generally not acidotic. They also reported that the phosphocreatine/adenosine triphosphate ratio was significantly lower compared to healthy brain tissue. Cadoux-Hudson *et al.'s* (3) study also showed elevated levels of phosphomonoester and phosphomonoester/adenosine triphosphate that were accompanied by reduced levels of phosphocreatine when compared to tissue spectra from seven controls (3). The reduction in phosphocreatine was interpreted as resulting from extensive necrosis (>20%) that was observed histologically. In this series, particularly low levels of phosphocreatine were measured within the tumor center, and low to normal levels in the tumor periphery. In agreement with this finding, Hubesch *et al.* (9) reported a significantly decreased phosphocreatine/inorganic orthophosphate ratio. These findings agree with our results that show the presence of phospholipids commonly associated with catabolic processes, and support the idea that low-energy processes may predominate in meningiomas.

In separate studies, both Hubesch *et al.* (9) and Brenton *et al.* (32) reported that PE was a major component of the phosphomonoester resonance band, and, as had been previously suggested, ribose-5-phosphate or adenosine monophosphate were not. This information, coupled with the results of a ^1H NMR spectroscopic study by Bruhn *et al.* (5), which showed a prominent choline signal in the spectra of these tumors as well as diminished levels of phosphodiester, would suggest that the relative concentrations of phosphoethanolamine and phosphocholine should be elevated in tissue extracts. These *in vivo* findings corroborate the need for a more detailed and specific *in vitro* study of the phospholipid composition of meningiomas.

Heindel *et al.* (7) compared ^{31}P NMR spectra of 13 meningiomas with spectra from "healthy tissue." The former showed obvious changes, including decreased levels of phosphocreatine below those of both the γ- and α-phosphates of adenosine triphosphate (ATP) but above that of β-ATP. The phosphodiester resonance was reduced in these tumors relative to normal tissue while the phosphomonoester resonance was occasionally elevated. Changes in the phosphomonoester resonance were thought to indicate the presence of phosphoethanolamine, a precursor and an indicator of active membrane biosynthesis within the tumor. Studies by Cerdan *et al.* (33) and Burt *et al.* (34) suggested that the phosphodiester resonance has substantial contributions from a small fraction of mobile phospholipids. It should be mentioned in this context that the phospholipids in eggs are detectable as fairly narrow resonances in high-resolution ^{31}P NMR, and that it is believed that these signals arise because the lipids are contained in mobile lipoprotein particles (35) that are similar to circulating plasma lipoproteins (36). Even if the phospholipids were sufficiently mobile to allow for signals to contribute to the phosphodiester resonance band *in vivo*, the ethanolamine phospholipids would not be substantial contributors to the phosphodiester band in meningiomas.

In human brain tumors examined by Horrocks and Sharma (37) for phospholipid and acyl group composition, the proportion of EPLAS as a percentage of total phospholipids was low and varied from 0.8 to 11.8%. The proportion of alkylacyl glycerophosphocholine was elevated in three out of five tumor types. The resonance at –0.78 ppm, which has been interpreted by Edzes *et al.* (27) to be due to alkylacyl glycerophosphocholine was only 0.1% in control specimens, but was elevated to 0.8–3.4% in tumors. By comparison, in our study alkylacyl glycerophosphocholine (–0.78 ppm) measured less than 1% of all phospholipid components. Cultured cells, and both primary and metastatic tumors, generally contain 2–3% of this component and 1–13% of EPLAS (35).

The phospholipids in malignant neural tumors have been studied using NMR and other analytical techniques. Many neoplastic tissues have been found to have markedly elevated levels of 1-alkyl-2,3-diacyl-*sn*-glycerols (38). This was proposed as pointing to a potential tumor marker; however, lower levels of this phospholipid found in hepatocellular carcinoma, and high levels induced by staph infections made this less tenable (38). In human brain tumors examined by Albert and Anderson (38) for phospholipid and acyl group composition, the proportions of EPLAS were found to be rather low, but varied from 0.8 to 11.8%. The proportion of alkylacyl glycerophosphocholine was elevated in three out of five tumor types. Choline plasmalogen (CPLAS) was only 0.1% in the control, but was elevated to 0.8–3.4% in the tumors. By comparison, in our study the CPLAS measured less than 1% in all tissues, while it has been noted that cultured cells, both primary and malignant, generally have 2–3% CPLAS and 1–13% EPLAS.

Ronen and Degani (39), using ^{13}C NMR spectroscopy on isolated cells, reported an increase in phosphomonoester metabolites in tumor cells when compared to the profiles of normal cells. Both phosphocholine and phosphoethanolamine were elevated in cancerous tissues, and, subsequent to therapy, the concentration of both of these species diminished. Other investigators have reported similar findings (11,40).

Daly and Cohen (11) found a consistent elevation of the choline and ethanolamine phosphomonoesters in a variety of neoplasms, including prolactinomas and metastatic lymphomas within the central nervous system, lung, breast, skin, liver and bone. Hubesch *et al.* (9) reported that the phosphomonoester resonance signal was elevated in cancerous tissues relative to other metabolites detected in the profile; however, the absolute concentration of both the phosphomonoester and phosphodiesters was lower in the tumors. Such a finding is consistent with the pronounced edema and presence of extracellular debris ordinarily found in tumors with degenerative processes where the presence of these extracellular components decreases the relative volume of viable cells. None of the six meningiomas studied by Daly and Cohen (11) showed a statistically significant rise in the concentration of the phosphomonoester. This finding is consistent with

the data obtained in our study regarding type 3 tissue spectra, including the "normal" or "near-normal" concentrations of ethanolamine phospholipids. Thomsen *et al.* (6) found no significant differences in phospholipid precursors in central nervous system tumors when compared with normal brain.

The identity of the unidentified phospholipids labeled CPLIP and U in Figure 2 is in question. CPLIP has been identified both as the choline plasmalogen (25), having an enol ether function at glycerol carbon-1, and as the corresponding alkylacyl glycerophosphocholine (27). However, these assignments are not certain because of the inability, thus far, of producing chemically pure standard samples. It is suspected (27) that the phospholipid labeled U is the corresponding alkylacyl glycerophosphoethanolamine, but this assignment is even less certain than that for the corresponding choline phospholipids. It should be noted, however, that the assignment of resonance U to alkylacyl glycerophosphoethanolamine is consistent with data reported for the chemical and spectroscopic properties of an unknown phospholipid at 0.13 ppm in eye tissues (41–45), as well as in other human tissues (46–48). The discovery of potentially new phospholipids is a significant advantage of the ^{31}P NMR approach.

In summary, *in vitro* ^{31}P NMR on phospholipid extracts from human intracranial meningiomas can provide phospholipid concentration profiles and can identify statistically significant differences among tumors and thus can differentiate between meningioma subgroups. The data corroborate and supplement *in vivo* spectroscopic findings on phospholipid metabolites and demonstrate the value of ^{31}P NMR phospholipid analyses on tissue extracts in the characterization of meningiomas of the central nervous system.

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METHOD

A Rapid Method for the Preparation of Ganglioside $G_{lac}2$ (GD3)

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A method is described for the preparation of ganglioside $G_{lac}2$ [(II^3(NeuAc)$_2$-LacCer, GD3] from cream of bovine milk using liquid-phase extraction with methanol or ethanol followed by anion exchange chromatography. The method is rapid and inexpensive; 1 kg cream, centrifuged from 14–15 L of bovine milk, yields approximately 70 mg of pure ganglioside $G_{lac}2$. The sialic acid constituent of ganglioside $G_{lac}2$ isolated from bovine milk cream consists solely of the *N*-acetylneuraminic acid derivative. The major components of its ceramide consist of octadecasphing-4-enine and the 22:0 (behenic acid) and 23:0 fatty acids.

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Early reports on the occurrence of ganglioside $G_{lac}2$ [II^3(NeuAc)$_2$-LacCer, GD_3) in the late sixties had suggested a wide distribution of this disialoglycosphingolipid in various tissues of many different animal species (2–5). $G_{lac}2$ is not only found cell-bound but is also contained in body fluids such as serum and milk, where it is probably conjugated to lipophilic carrier proteins (6,7) (see also Table 1).

In spite of its disialogrouping, $G_{lac}2$ has a simple structure as compared, for example, to the higher brain gangliosides. It may, therefore, be considered a developmental precursor of the latter, more complex sialoglycolipids. Ganglioside $G_{lac}2$, and cells binding the $G_{lac}2$-specific monoclonal antibody R24 have been found in cell types at later embryonic stages and in adult tissues and tumors that may indicate some neuroectodermal association of this sialoglycolipid. A possible functional role of $G_{lac}2$ was pointed out for epithelial-mesenchymal cell interactions (8).

In a variety of cancers, ganglioside $G_{lac}2$ and its 9-*O*-acetyneuraminyl derivatives accumulate, possibly as a consequence of the cells' inability to synthesize the respective higher analogs (see Table 1). In addition, cancer cells are believed to shed tumor-associated glycolipids, that in turn may contribute to increased serum levels of ganglioside $G_{lac}2$ as suggested for human melanoma (9), glioma (10) and, possibly, alteration of the immune status of melanoma patients (11–13).

An involvement of ganglioside $G_{lac}2$ in cell surface interactions and cell adhesion has recently been postulated (8,14,15). Ganglioside $G_{lac}2$ together with its 9-*O*-acetylneuraminyl or lactone derivatives is also suspected of playing a functional role in the development of the nervous system.

In view of the putative roles of ganglioside $G_{lac}2$ in various tumor-associated phenomena as well as other biological interactions, it appears likely that a demand for this glycolipid will arise in the future. We have therefore developed a simple and inexpensive method for the convenient isolation of $G_{lac}2$. The procedure we describe for the preparation of pure ganglioside $G_{lac}2$ uses cream from bovine milk as a safe and readily available source and methanol or ethanol as a solvent for the liquid-phase extraction of the sialoglycolipid.

MATERIALS AND METHODS

Extraction of ganglioside. To 1 kg of cream from fresh, nonhomogenized bovine milk is added two liters of methanol, and the mixture is stirred mechanically for 15 min at room temperature. After centrifugation for 30 min at 3,000 × *g* at 4°C, the clear supernatant is decanted and the pellet residue reextracted twice, each time with 2 L of methanol. The three extracts obtained contain 32, 60 and 8% of the total extracted ganglioside $G_{lac}2$, respectively. Further continuation of extraction did not significantly increase the yield of ganglioside. Instead of methanol, ethanol may be used for extraction with similar efficiency.

The three extracts are then sequentially passed over a DEAE-Sephadex A25 column (Pharmacia, Uppsala, Sweden; r = 1.75 cm, l = 25 cm, vol, 250 mL) that had been prewashed with methanol. All gangliosides are thereby retained and can be eluted under standard conditions. The column is then eluted sequentially with 2 L of methanol (eluting neutral compounds) and 2 L of 0.02 M methanolic ammonium acetate [eluting the predominant portion of the monosialogangliosides with $G_{lac}1$ (GM3) as the major compound]. The ganglioside $G_{lac}2$ is eluted from the column with 2 L of 0.2 M methanolic ammonium acetate. After reducing the solvent to approximately 100 mL by rotary evaporation, the fraction is then dialyzed for 48 h against 5 L of distilled water with five changes of the dialysis medium. Finally, the fraction is evaporated to dryness by rotary evaporation.

Final purification of ganglioside $G_{lac}2$. The disialoganglioside fraction containing trace amounts of monosialoganglioside $G_{lac}1$ (see Fig. 2, fractions I/H/W 55:37:8-2 and 55:36:9-1) besides 9-*O*-acetylated $G_{lac}2$ (see Fig. 2, fractions 55:36:9-2, 55:36:9-3 and 55:35:10-1) is dissolved in *iso*propanol/*n*-hexane/water (I/H/W; 55:40:5, by vol) and applied to a silica gel column (Silica Gel 60; Merck, Darmstadt, Germany; r = 1.5 cm, l = 28 cm; vol, 200 mL). For ganglioside separation a stepwise gradient of I/H/W containing 5 to 10% water is used. The gradient consisted of 800 mL each of I/H/W

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Abbreviations: DEAE, diethylaminoethyl; I/H/W, *iso*propanol/*n*-hexane/water; TLC, thin-layer chromatography.

Short hand notations for gangliosides are according to Wiegandt (Ref. 1). $G_{lac}2$, (II^3(NeuAc)$_2$-LacCer) is NeuAcα,8NeuAcα,-3Galß,4Glcß,1Cer; $G_{tet}1$, (II^3NeuAc-Gg_4Cer) is Galß,3GalNAcß,-4(NeuAcα,3)Galß,4Glcß,1Cer.

(55:40:5 and 55:29:6, by vol); 400 mL each of I/H/W (55:38:7, 55:37:8 and 55:36:9, by vol), and 1200 mL of I/H/W (55:35:10, by vol). The fractions I/H/W (55:40:5–55:38:7, by vol) containing the major portion of the phospholipids are discarded. The eluate obtained with I/H/W (55:37:8, by vol) is collected in two, the I/H/W (55:36:9, by vol) eluate in three and the I/H/W (55:35:10, by vol) eluate in six consecutive fractions. The latter six fractions were free of detectable phospholipids and contained pure ganglioside $G_{lac}2$ (Figs. 1 and 2).

Identification of ganglioside $G_{lac}2$ sialic acid. $G_{lac}2$ (50 µg) in 200 µL 0.05 M aqueous HCl is heated to 70°C for 60 min. After removal of HCl by repeated evaporation with water, the hydrolysate is applied to a small column of 200 µL silica gel RP-18. Sialic acid is eluted with 1 mL water. The eluate is then brought to dryness, and the sialic acid identified by chromatography on cellulose high performance thin-layer chromatography (TLC) plates (Merck). The plates are pre-run with 0.1 M HCl and dried overnight in a desiccator over potassium hydroxide/phosphorus pentoxide. The probes are then applied to the plates and chromatographed using *n*-butanol/*n*-propanol/0.1 M aq. HCl (1:2:1, by vol). Sialic acid is visualized with Ehrlichs reagent, i.e., spraying with 0.6% dimethylaminobenzaldehyde in ethanol/37% aq. HCl (8:2, vol/vol), and heating to 110°C for approximately 10 min beneath a preheated glass plate.

RESULTS AND DISCUSSION

Ganglioside $G_{lac}2$ is widely distributed in cells and body fluids of vertebrates. Among these, bovine milk has been reported to be particularly rich in this sialoglycosphingolipid (Table 1). Ganglioside $G_{lac}2$ has, therefore, been prepared previously by several workers from milk (7) or buttermilk (16–18). Bovine milk was shown to contain 5.64 µmol/L of ganglioside $G_{lac}2$, of which 92.3 and 7.5% was present in the cream and the buttermilk, respectively (7). We therefore chose the cream fraction as starting material for the ganglioside $G_{lac}2$ preparation.

The first step in the previously described ganglioside $G_{lac}2$ isolation procedures (16–18) involved the evaporation of water from the starting material and the subsequent extraction of the dry powder with organic solvents, tetrahydrofuran (16,19), or in all other cases chloroform/methanol.

The innovation introduced in our present procedure avoids these steps, i.e., the drying-down of the starting material, the use of costly and potentially hazardous solvents, and a heterogeneous phase extraction. Instead, bovine milk cream is treated in homogeneous phase with dilute methanol, and the extraction residue formed thereby is separated by low-speed centrifugation or filtration. The gangliosides are isolated by simply passing the methanol extract over an anion-exchange column. The retained gangliosides are subsequently obtained by stepwise elution according to standard procedures. The final purification of the ganglioside $G_{lac}2$ involves silica gel column chromatography under standard conditions.

Using this procedure, from 1 kg cream (prepared by centrifugation of 14–15 L fresh bovine milk, 30 min, 3,000 × *g*, 4°C) 70 mg of pure ganglioside $G_{lac}2$ (53.8 µmol ≈ a yield of 3.8 µmol/L milk; Fig. 1) was isolated. Ganglioside yield determinations were by TLC and subsequent quantification by resorcinol/HCl staining and scanning at 580 nm with a Shimadzu TLC scanner CS-9000 (Tokyo, Japan). The compositional analysis of the $G_{lac}2$ lipid moiety showed octadecasphing-4-enine (d18:1) and 23:0 fatty acid as well as behenic acid (22:0) to be the major ceramide constituents (Table 2). This

TABLE 1

Distribution of Ganglioside $G_{lac}2$

Species	Source	Cell type	Reference
Human	Brain		(20,21)
	Retina		(2,22)
	Thyroid		(23)
	Prostate tissue		(24)
	Semen		(25)
	Serum		(6)
	Brain tumors		(26,27)
	Melanoma		(28–31)
	Glioblastoma		(32)
	Meningioma		(33)
	Leucocytes		(34)
Bovine	Retina		(2,22,35)
	Spleen		(4)
	Liver		(4,36)
	Udder		(4,7)
	Buttermilk		(16–18,37)
	Milk		(7)
Cat	Erythrocytes		(5)
Chick	Embryo		(38)
Mouse	Brain		(39)
	Neural crest	Neurons	(40)
Pig		Erythrocytes	(41)
		Lymphocytes	(42)
Rabbit	Retina		(22)
	Thymus		(43)
Rat	Retina		(44,45)
	Adrenals		(46)
	Dorsal root ganglia		(47)

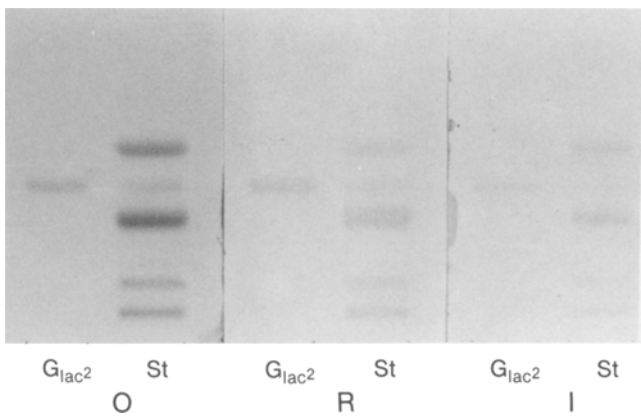

FIG. 1. Thin-layer chromatogram of ganglioside $G_{lac}2$ from bovine milk-cream. Running solvent, chloroform/methanol/0.2% $CaCl_2$, 45:45:10 (by vol); $G_{lac}2$, 2 µg were applied to the plate; St, standard brain gangliosides; O, orcinol/H_2SO_4 staining; R, resorcinol/HCl staining; I, iodine vapor staining.

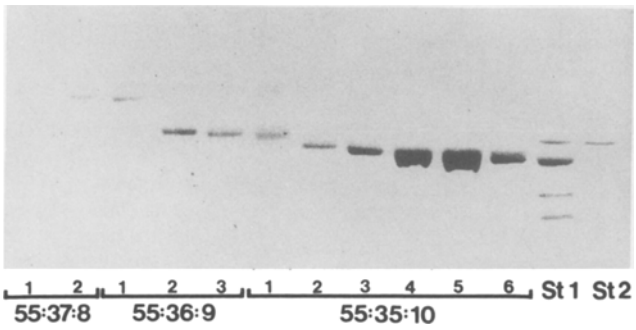

FIG. 2. Thin-layer chromatographic analysis of the fractions obtained by silica gel chromatography of bovine milk cream disialogangliosides. Abscissa: elution by *iso*propanol/*n*-hexane/water (I/H/W) in the proportions given (numbers 1 to 6, indicate consecutively collected fractions); St 1, standard brain gangliosides; ST 2, standard ganglioside $G_{tet}1$. Fractions I/H/W 55:37:8/-2 and 55:36:9/-1, $G_{lac}1$; 55:36:9/-2,3 and 55:35:10/-1, 9-*O*-acetyl-$G_{lac}2$; 55:35:10/-2,3,4,5,6, $G_{lac}2$. Detection was with resorcinol/HCl spray reagent.

finding is in general agreement with a previous report (17). The isolation requires approximately nine one-person working days.

Ganglioside $G_{lac}2$ prepared from bovine milk cream contained exclusively *N*-acetylneuraminic acid as its sialic acid constituent. *N*-glycolylneuraminic acid, if present, was below detection limits. Even though, in addition to *N*-acetylneuraminic acid, the presence of the *N*-glycolyl derivative has been described for gangliosides of bovine mammary gland and milk (7), our findings would be consistent with the notion that only the *N*-acetyl variant is present in gangliosides of bovine milk (16,17).

TABLE 2

Sphingoid and Fatty Acid Composition of Ganglioside $G_{lac}2$ from Bovine Milk Cream[a]

Fatty acid		Sphingoid	
16:0	3.4	d16:1	10.9
18:1	3.7	d16:0	5.3
18:0	8.8	t16:0	7.7
20:0	3.2	d18.1	52.6
21:0	3.0	d18.0	6.5
22:0	26.4	d19:1	5.0
23:0	28.8	d21:1	5.9
24:1	3.2	t22.0	6.1
24:0	16.7		
25:0	2.9		

[a]Data are given in percentage of the total. Sphingoid was analyzed by gas chromatography/mass spectrometry after hydrolysis of $G_{lac}2$ (Ref. 48), isolation of the long-chain base (Ref. 49), and oxidation with lead tetraacetate of the aldehydes produced (Ref. 50). Fatty acids were determined as their methyl esters by gas chromatography.

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COMMUNICATION

Cytoprotective Effect of Tocopherols in Hepatocytes Cultured with Polyunsaturated Fatty Acids

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When highly unsaturated fatty acids are added to cell cultures, it can become important to include antioxidants in the culture medium to prevent cytotoxic peroxidation. To find an optimal antioxidant for this purpose, the effect of 50 μM α-tocopherol, γ-tocopherol, α-tocopheryl acetate, α-tocopheryl acid succinate, or α-tocopheryl phosphate, or of 1 μM *N,N'*-diphenyl-1,4-phenylenediamine, was investigated with respect to the agent's ability to prevent lactate dehydrogenase leakage in long-term rat hepatocyte cultures supplemented with 0.5 mM highly unsaturated fatty acids. Formation of thiobarbituric acid reactive substances in the cultures was also measured. α-Tocopheryl acid succinate was found to be the most effective cytoprotective compound, followed by *N,N'*-diphenyl-1,4-phenylenediamine, α-tocopherol, γ-tocopherol and α-tocopheryl acetate, and α-tocopheryl phosphate was without effect.

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When studying the effect of various highly unsaturated fatty acids (HUFA) in cell cultures, it can become desirable to prevent cytotoxic effects due to lipid peroxidation. α-Tocopherol (α-T) is the major lipid-soluble, chain-breaking antioxidant in biological systems (1) and has been shown to protect membranes from polyunsaturated fatty acids and lysophospholipids (2). Esters of α–T are commonly added to cell culture media, as these esters probably are more readily taken up by the cells than is α-T, and are then hydrolyzed by esterases, thereby releasing the active α-T. The synthetic antioxidant *N,N'*-diphenyl-1,4-phenyl-enediamine (DPPD) has also been shown to have vitamin E-like activity (1).

We have previously reported that supplementing rat hepatocytes in long-term culture with 0.5 mM HUFA resulted in leakage of intracellular lactate dehydrogenase (LDH) (3). This cytotoxic effect could be attenuated by supplementing the medium with 50 μM α-tocopheryl acid succinate (TS) or α-tocopheryl phosphate (TP), the former being more effective than the latter (3). In the present study we have investigated a number of tocopherol derivatives as well as DPPD for their effect on LDH-leakage and formation of thiobarbituric acid reactive substances (TBARS) in long-term rat hepatocyte cultures supplemented with 0.5 mM arachidonic acid.

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Abbreviations: DMSO, dimethylsulfoxide; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; HUFA, highly unsaturated fatty acids; LDH, lactate dehydrogenase; MDA, malondialdehyde; α-T, α-tocopherol; γ-T, γ-tocopherol; TA, α-tocopheryl acetate; TBARS, thiobarbituric acid reactive substances; TP, α-tocopheryl phosphate; TS, α-tocopheryl acid succinate.

MATERIALS AND METHODS

Reagents. Enzymes, coenzymes, collagen, horse serum, hormones and fatty acids were prepared or obtained as described previously (3). DPPD and ferric chloride hexahydrate were obtained from Aldrich Chemie (Steinheim, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Hepatocytes were isolated from 48-h starved female Wistar rats, weighing 210–250 g before starvation, and the cells were cultured in a modified Waymouth medium (4) as described earlier (3). A starvation period of 48 h was chosen as this increased the susceptibility of the cultured cells to the effects of HUFA as compared to cells from 16-h starved rats (3). The hepatocytes (approximately 45 μg DNA per dish) were maintained in culture for 72 h, with the medium being changed every 24 h. During the last 48 h, the medium was supplemented with 1% (wt/vol) fatty acid-free albumin, 0.1 μM insulin, 0.1 μM dexamethasone, 0.5 mM fatty acid and antioxidants. The following antioxidants were used (final concentration, solute): DPPD [1 μM, dimethylsulfoxide (DMSO)]; (±)α-T (50 μM, acetone); (+)-γ-tocopherol (γ-T) (50 μM, acetone); (±)-α-tocopheryl acetate (TA) (50 μM, acetone); (±)-TP (50 μM, water) and (+)-TS (50 μM, DMSO). The concentrations of DMSO and acetone added to the media, 0.46% (vol/vol) and 0.1% (vol/vol), respectively, had no effect on any of the parameters measured. Specific solvents were chosen to facilitate solubilization of the agents in the aqueous phase. The solubilization of the tocopherols was confirmed by measuring the absorbance between 284 and 292 nm in the culture media after tenfold dilution.

Peroxidation of arachidonic acid during the preparation and storage of fatty acid-containing media, even at 37°C, was negligible as TBARS measurements in media with and without arachidonic acid gave similar values, i.e., about 1.7–2.0 μM TBARS (n = 1).

Determination of LDH leakage and DNA content. LDH-leakage and DNA content were measured as described earlier (3). In short, cells and medium were separated, the cells were homogenized by ultrasonication, and aliquots of cell homogenate and medium were analyzed for LDH activity by measuring NADH formation at 340 nm. The DNA content in the cell homogenate was measured fluorimetrically. Cell viability was measured as percentage of LDH released into the medium.

Determination of TBARS. Lipid peroxidation was expressed as TBARS accumulated in the medium (5). TBARS were determined as described previously (3), in the presence of ferric chloride and butylated hydroxytoluene while heating (6), and by measuring absorbance at 532 nm (7). After subtraction of the background, due to

the phenol red in the culture medium, absorbance was converted to TBARS and expressed in malondialdehyde (MDA) equivalents; a standard curve for MDA was generated based on hydrolyzed 1,1,3,3-tetraethoxypropane. Lipid peroxidation was expressed as nmol TBARS (MDA equivalents)/mg DNA.

Statistical evaluation. Statistical significance of differences was assessed by analysis of variance; the Scheffe test was used for comparison of means. $P < 0.05$ was considered statistically significant.

RESULTS

In this study, cytotoxicity was defined as the increase in LDH-leakage seen upon exposure of the cells to fatty acid. The LDH in the culture medium originates from hepatocytes that have lost their membrane barrier properties; the LDH that leaks into the medium is typically not degraded within 24 h (8).

Figure 1 illustrates that fatty acids with more than three double bonds cause greater LDH-leakage and TBARS formation than less unsaturated fatty acids. The addition of TS caused a significantly lower LDH leakage compared to TP, when the cells were incubated with linoleic acid, columbinic acid, arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid (Fig. 1A). A similar pattern was seen for the production of TBARS with columbinic acid, arachidonic acid and eicosapentaenoic acid (Fig. 1B). Measurements of LDH-leakage 2 h after fatty acid addition to the cultures indicated that none of the fatty acids were cytotoxic by themselves in our experimental system (1–3% LDH-leakage, n = 7).

The cytoprotective effect of 50 μM α-T, γ-T, TA, TP, TS and 1 μM DPPD in cultures supplemented with 0.5 mM arachidonic acid is shown in Figure 2. TS, α-T, γ-T and DPPD had similar effects on LDH leakage, whereas a significantly smaller protective effect was seen with TA and TP. The production of TBARS showed a similar pattern, as did LDH-leakage (Fig. 3).

The dose-response relationship between antioxidant concentration and LDH-leakage was tested in the presence of 0.5 mM arachidonic acid in the range from 25 to 200 μM tocopherols and 1 to 50 μM DPPD. Only TP showed a dose-response relationship, i.e., increasing concentrations of TP gave an increase in LDH-leakage, suggesting a cytotoxic effect of TP at higher doses (data not shown, n = 3). Cultures incubated with antioxidants (tocopherols, 50 and 200 μM, DPPD, 1 and 50 μM) and without fatty acid showed that none of the tocopherols were cytotoxic at 50 μM, whereas addition of 200 μM TP or TS resulted in an LDH-leakage of 66 and 35%, respectively (mean, n = 3). DPPD showed no cytotoxic effects in the concentration range studied.

DISCUSSION

HUFA at 0.5 mM was found to be cytotoxic in our *in vitro* cell system, an effect that was thought to be due to lipid peroxidation (3). A similar cytotoxic effect of exogenous HUFA has previously been seen in other cell culture systems (9,10).

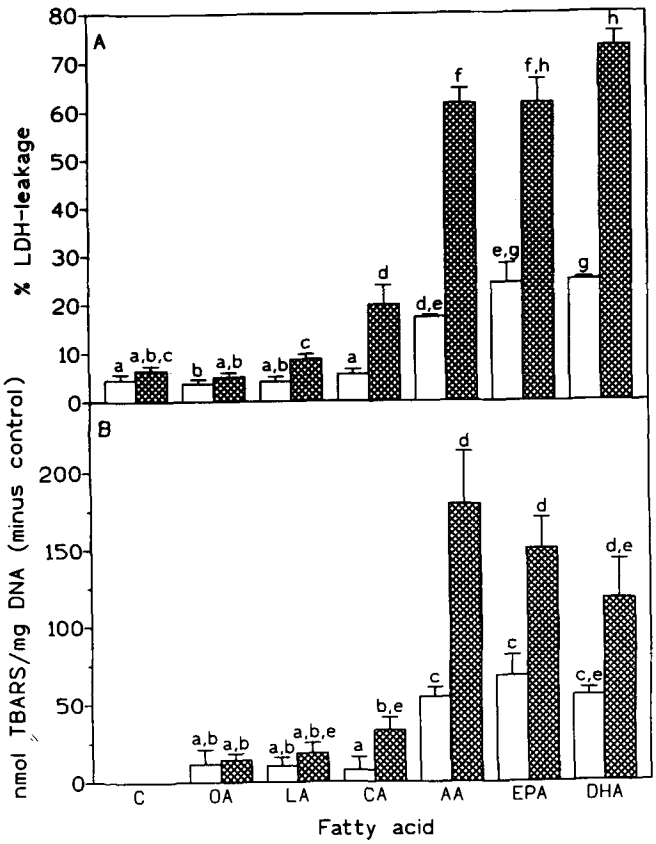

FIG. 1. The cytoprotective effect of α-tocopheryl acid succinate (TS) and α-tocopheryl phosphate (TP) in cultures supplemented with various polyunsaturated fatty acids. (A) % Lactate dehydrogenase (LDH)-leakage; (B) formation of thiobarbituric acid reactive substances (TBARS). Hepatocyte monolayers were prepared from 48-h starved rats and maintained in culture for 48 h in the presence of 50 μM TS (open bars) or 50 μM TP (cross-hatched bars) and 0.5 mM oleic acid (OA), linoleic acid (LA), columbinic acid (CA), arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). (A) C is the control without fatty acid and antioxidant. Each bar represents the mean ± SE of four separate cultures. (B) The control value (134–177 nmol TBARS/mg DNA) was subtracted. Each bar represents the mean ± SE of three separate cultures. Values with different letters are significantly different.

In the present study, the cell cultures were heavily loaded with tocopherols (50 μM tocopherol derivative, or 150 nmol per culture dish containing *ca.* 10^6 cells). Nevertheless, none of the antioxidants tested could fully prevent HUFA-induced LDH-leakage. This, and the lack of a correlation between LDH-leakage and TBARS production (Figs. 1–3), would suggest that HUFA cytotoxicity is partly caused by mechanisms that are not related to tocopherol-suppressible lipid peroxidation. It has been seen in many cases that inhibition of lipid peroxidation does not prevent oxidative stress-induced cell injury (11). Fariss (5) and Carini *et al.* (12) reported TS to be a better antioxidant than α-T in hepatocyte suspensions, where oxidative stress was induced by an atmosphere of 95% O_2, 5% CO_2 and the presence of adenosine diphosphate/iron, cumene hydroperoxide or carbon tetrachloride.

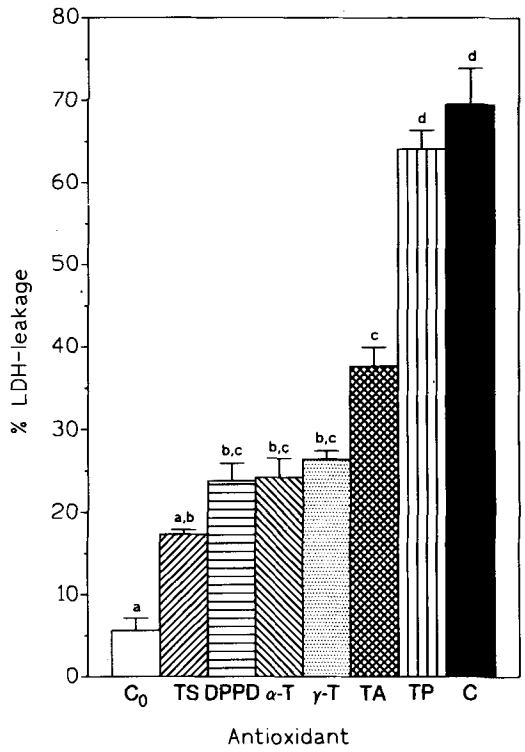

FIG. 2. The cytoprotective effect of various antioxidants in cultures supplemented with arachidonic acid. Hepatocyte monolayers were prepared from 48-h starved rats and maintained in culture for 48 h with 0.5 mM arachidonic acid and 50 μM of tocopherol or 1 μM DPPD added. C_0 is the control without arachidonic acid and without antioxidant; C is the control with arachidonic acid and without antioxidant. Each bar represents the mean ± SE of three separate cultures. Values with different letters are significantly different. DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; α-T, α-tocopherol; γ-T, γ-tocopherol; TA, α-tocopheryl acetate. Other abbreviations in Figure 1.

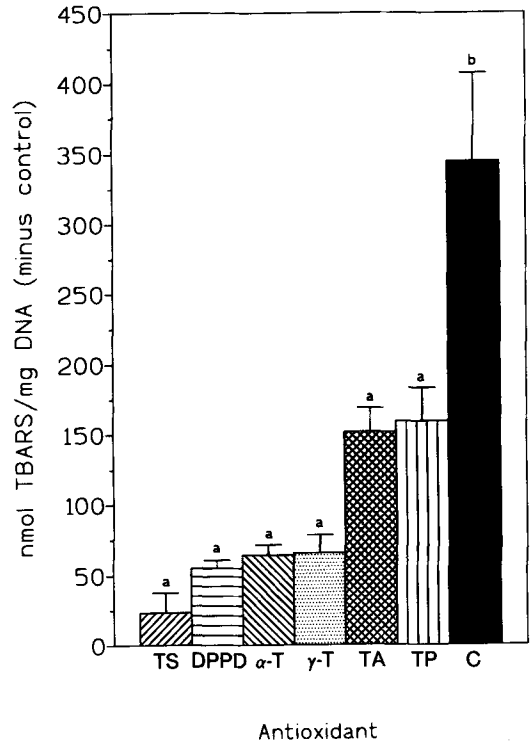

FIG. 3. The effects of various antioxidants on the formation of TBARS. Hepatocyte monolayers were prepared from 48-h starved rats and maintained in culture for 48 h with 0.5 mM arachidonic acid and 50 μM of tocopherol or 1 μM DPPD added. C is the control with arachidonic acid and without antioxidant. The control values without arachidonic acid and antioxidant (168–196 nmol TBARS/mg DNA) were subtracted. Each bar represents the mean ± SE of three separate cultures. Values with different letters are significantly different. Abbreviations as in Figures 1 and 2.

It is reasonable to suggest that the different tocopheryl esters need to be hydrolyzed by esterases and that the actual cytoprotective effect is exerted by α-T. TS has been suggested to act as a carrier of α-T enabling the release and accumulation of α-T at specific and critical cellular sites (12). The different effects of the tocopherols and their esters may thus reflect differences in transmembrane mobility and in the rates of ester hydrolysis by intracellular esterases. α-T may also have a lower rate of transmembrane mobility than TS, as α-T may exist in micellar form in the medium (13). In addition, the tocopheryl esters are hydrolyzed at different rates by membrane-bound esterases (14). The succinate moiety of TS may have an additional effect, consistent with the proposal by Maguire *et al.* (15), who showed that submitochondrial particles containing the succinate–ubiquinone reductase complex protected α-T from consumption which may suggest the reduction of the α-tocopheryl radical to α-T.

In summary, we have shown that it is important to supplement culture media with antioxidant and to monitor cell viability when culturing cells with exogenous HUFA. The tocopherols we investigated differ significantly in their cytoprotective effect, with TS being the most effective.

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Thermal Adaptation Affects the Fatty Acid Composition of Plasma Phospholipids in Trout

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The adaptive changes in the fatty acid (FA) composition of plasma phospholipids (PL) in response to alterations in environmental water temperature were investigated in juvenile rainbow trout *(Oncorhynchus mykiss)*. The changes observed during thermal adaptation from 22°C in summer to 8°C in winter were reproduced by laboratory cold acclimation (CA) at 6°C of 22°C-summer-acclimated animals. In cold-acclimated and winter-acclimated trout, the increase in the unsaturation of PL fatty acids was mainly due to an enrichment of approximately 7% in the total weight percentage of 22:6n-3, while a concomitant significant decrease in the levels of 18:0 and of the monoenoic n-9 FA was observed. A time course study revealed that the changes in PL fatty acids became significant after 10 d of CA and were complete after one month. These changes in the composition of the fatty acyl chains of plasma total PL indicate that the FA composition of plasma lipoprotein PL does not remain constant during thermal adaptation. This would suggest that plasma lipoproteins provide a rapid systemic supply of lipids containing more or less unsaturated FA during thermal adaptation of poikilothermic animals.

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The metabolic changes that occur during thermal adaptation of poikilothermic animals cause alterations in membrane lipid composition that assure the maintenance of normal membrane functions under thermal stress. This homeoviscous adaptation (1) to environmental temperature is responsible for maintaining membrane fluidity and function at low temperatures. It involves restructuring of the polar headgroup composition of phospholipid (PL) classes, together with modification of the unsaturation of their fatty acyl chains (2–6).

Salmonids are eurythermic fish that undergo temperature variations in their natural environment (7). In these fish, PL have a complex fatty acid (FA) pattern characterized by a large proportion of polyunsaturated fatty acids (PUFA) (8). The renewal and restructuring of membrane lipids following thermal adaptation could involve plasma lipids and lipoproteins, since the capacities for lipogenesis and FA desaturation vary from one tissue to another. Numerous studies in different fish species (for a review, see Ref. 3, and also Refs. 9–11) have demonstrated structural and composition changes of membrane PL following thermal adaptation but there are no data on the effect of temperature changes on the FA composition of plasma lipids in poikilotherms. A circannual variation in the FA composition of plasma and high density lipoprotein PL have been recently demonstrated in rainbow trout (12), a teleost fish that remains active at low temperatures.

The objectives of the present study were to investigate in trout the effects of thermal adaptation in the laboratory on the composition of plasma PL fatty acids and to compare the changes induced to those observed in response to seasonal temperature variations.

MATERIALS AND METHODS

Animals. Juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes were used. The animals were offsprings of a single male and female and were exposed to the natural photoperiod throughout the experiment. Before the experiments, the trout were nourished *ad libitum* three times a week with a standard commercial granule diet (Aqualim, Nersac, France) containing 11% lipids. The diet composition was held constant regardless of the water temperature at which the animals were kept. Trout were anesthetized with ethylenglycol monophenyl ether (0.3 mL/L). Blood was removed over ethylenediaminetetraacetic acid disodium salt and NaN_3 (3 and 0.15 mg/mL blood) dissolved in 0.15 M NaCl, final pH 7.4, by cardiac puncture with a fine catheter (diameter 0.58 mm) and kept at 4°C throughout the procedure. Plasma was obtained by centrifugation (3000 × *g*, 10 min).

Experimental design. Juvenile trout, 1½-years-old, acclimated to 22°C in August, were randomly divided into two groups. Ten days after the first blood sampling, the first group was subjected to cold acclimation (CA) at 6°C by progressively decreasing water temperature from 22 to 6°C within 20 h. The second group was subjected to summer variation of water temperature from 22 to 21°C. Blood was drawn at an interval of 4 or 7 d between successive samples over a period of 41 d. Trout were given free access to food at least 4 h after each sampling and were left unfed until the next sampling. Furthermore, in order to determine the effects of seasonal thermal adaptation on plasma PL fatty acids, marked juvenile trout were sampled in summer at 22°C and in winter at 8°C.

FA analysis. FA composition of plasma PL was determined as previously described (13). Lipid standards or 10 µL of plasma were applied directly to the preadsorbent zones of thin-layer silica gel plates (G-1520, 20 × 20 cm; Schleicher & Schuell, Dassel, Germany). The thin-layer chromatography plates were partially developed with methanol followed by chloroform/methanol (1:1, vol/vol) to approximately 1.5 cm above the preadsorbent border. The plates were then fully developed to 15 cm above the adsorbent band in hexane/diethyl

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Abbreviations: ANOVA, analysis of variance; CA, cold acclimation; FA, fatty acids; HDL, high density lipoproteins; PL, phospholipids; PUFA, polyunsaturated fatty acids; UI, unsaturation index; U/S, ratio of unsaturated to saturated fatty acids.

ether/acetic acid (80:20:1, by vol) to separate the major lipid classes. The resulting bands were located by spraying the plate with rhodamine 6 G (0.07% in 95% ethanol) and viewing under ultraviolet light. Fractions corresponding to PL were scraped into screw-capped tubes, and treated with boron trifluoride/methanol as previously described (13). Heptadecanoic acid (17:0) and tricosanoic acid methyl ester (23:0 Me) were used as internal standards. FA methyl esters were analyzed with a Carlo Erba 4100 gas chromatograph (Milan, Italy), equipped with a Ross needle type injector and a flame-ionization detector, coupled to an ENICA 10 integrator (Delsi, Suresnes, France). The silica capillary column (25 m × 0.32 mm i.d.) was coated with OV-1 (Chrompack, Middelburg, The Netherlands). The column temperature was programmed from 150 to 180°C at 5°C per min after an initial 25 min at 150°C, and was held at 180°C for 60 min. Hydrogen was used as carrier gas at a pressure of 0.7 bar. FA methyl esters were identified by comparison of their relative retention times to those of known standards. A small amount of arachidonic acid (20:4n-6) methyl ester was present but was not resolved from the eicosapentaenoic acid (20:5n-3) methyl ester on the OV-1 capillary column. The unsaturated to saturated FA ratio (U/S) was calculated from the total weight percentages of monoenoic FA + PUFA/total weight percentages of saturated FA. The unsaturation index (UI) of plasma PL FA, defined as the average number of double bonds per FA, was calculated as the sum of the weight percentage multiplied by the number of double bonds for each FA in the mixture/100.

Statistics. Data are presented as means ± SEM and were tested for statistical significance by analysis of variance (ANOVA) followed by an evaluation employing Tukey-Kramer's multiple range test. For comparisons between FA weight percentages, data subjected to ANOVA were transformed (arcsin) prior to statistical analysis. Comparisons between summer- and winter-acclimated trout or summer-acclimated and cold-acclimated trout were done by two-tailed Student's *t*-test. Results were evaluated with the Statistical Analysis System (SAS) computer programs (14). The *P* value chosen for statistical significance was 0.05.

RESULTS

Plasma PL of 8°C-winter-acclimated or 6°C-acclimated trout contained larger quantities of docosahexaenoic acid (22:6n-3) and smaller quantities of oleic acid (18:1n-9) and gondoic acid (20:1n-9) than 22°C-summer-acclimated animals (Table 1). A significant reduction in the level of stearic acid (18:0), the saturated FA precursor of the n-9 family, also occurred during cold adaptation (Table 1). The time courses of the changes in the weight percentages of the two major unsaturated FA of trout plasma PL, 18:1n-9 and 22:6n-3, during CA are shown in Figure 1. After 6 d of CA, there was a significant decrease in the percentage of 18:1n-9 in plasma PL (10.63 ± 0.24 vs. 13.75 ± 0.64%, $P < 0.05$), which became highly significant after 10 d of CA (9.24 ± 0.13%, $P < 0.001$). The proportion of 22:6n-3 evolved inversely to that of 18:1n-9. The increase in 22:6n-3 became very significant after 17 d of CA (37.06 ± 1.34 vs. 31.41 ± 0.63%, $P < 0.005$) and was similar to that of 8°C-winter-acclimated trout (36.51 ± 1.25%). The increase in the proportion of 22:6n-3 during seasonal thermal adaptation from 22 to 8°C or during CA to 6°C corresponds to an enrichment of this PUFA in plasma PL of approximately 7% (Table 1).

TABLE 1

Fatty Acid Composition of Plasma Phospholipids from Summer-Acclimated, Winter-Acclimated and Cold-Acclimated Juvenile Trout[a]

Fatty acid	Summer (22°C)	Winter (8°C)	Cold-acclimated (6°C)
14:0	1.66 ± 0.15	1.84 ± 0.07	2.15 ± 0.23
16:0	25.38 ± 0.64	24.79 ± 0.83	26.53 ± 0.61
18:0	10.31 ± 0.50	6.46 ± 0.71[d]	5.89 ± 0.18[e]
16:1n-7	3.38 ± 0.18	3.12 ± 0.10	2.68 ± 0.43
18:1n-9	11.88 ± 0.42	10.02 ± 0.71[b]	8.96 ± 0.37[d]
20:1n-9	2.01 ± 0.04	1.48 ± 0.14[c]	1.38 ± 0.07[e]
18:2n-6	2.47 ± 0.10	2.31 ± 0.16	2.50 ± 0.07
20:3n-6	1.47 ± 0.18	1.37 ± 0.13	1.38 ± 0.14
20:5n-3	8.94 ± 0.35	10.01 ± 0.90	8.27 ± 0.39
22:5n-3	2.05 ± 0.15	2.10 ± 0.10	2.14 ± 0.09
22:6n-3	30.53 ± 0.77	36.51 ± 1.25[d]	38.16 ± 1.83[d]
U/S	1.65 ± 0.01	1.99 ± 0.04[e]	1.89 ± 0.05[d]
UI	2.61 ± 0.04	2.99 ± 0.05[d]	2.99 ± 0.02[e]

[a]Values are means ± SEM, n = 5 in each group. Data are presented as weight percentages. Only the major fatty acids are listed (percentage values >1%). Marked juvenile trout were blood sampled in summer at 22°C and in winter at 8°C. In order to determine the effects of low-temperature adaptation on plasma phospholipids fatty acids, trout acclimated to 22°C in summer were subjected to cold acclimation by decreasing water temperature from 22 to 6°C. Trout were cold-acclimated to 6°C during 31 d before blood sampling. The significance for the differences between 2°C-summer- and 8°C-winter-acclimated trout was tested with the paired two-tailed Student's *t*-test. Comparisons between 22°C-summer-acclimated and 6°C-cold-acclimated trout were by unpaired two-tailed Student's *t*-test. U/S, ratio of unsaturated to saturated fatty acids; UI, unsaturation index.

[b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.005$; [e]$P < 0.0001$.

The net result of these compositional changes was a significant increase in the U/S ratio at low temperatures (Table 1). The elevated proportion of 22:6n-3 during CA or during seasonal adaptation from summer to winter resulted in an increase in the UI of FA at low temperatures (Table 1 and Fig. 2). This index increased very significantly after 10 d of CA (2.90 ± 0.03 vs. 2.62 ± 0.09, $P < 0.005$) and became identical to that of winter-acclimated trout after 31 d of CA (Table 1 and Fig. 2). At that time, the UI of the acyl chains was very different from that calculated for the PL of summer-acclimated trout (Table 1).

DISCUSSION

The maintenance of biomembrane functions in poikilotherms, regardless of the environmental temperature, involves changes in the lipid composition. At low temperatures, the modifications involve changes that result in an increase in the degree of FA unsaturation in dif-

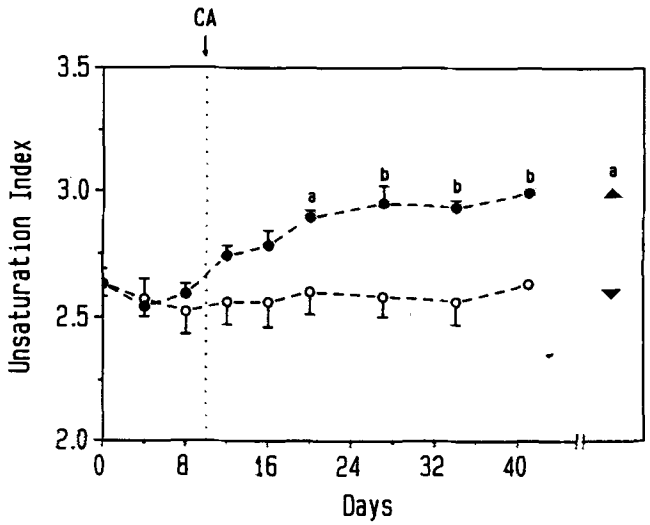

FIG. 1. Temporal changes in the weight percentage of 22:6n-3 (circle) and 18:1n-9 (square) in plasma phospholipid molecular species, in cold-acclimated (●,■) and in summer-acclimated juvenile trout (○,□). For details in experimental design, see Materials and Methods. Values are means ± SEM, n = 5 (SEM within data points are not shown). For each group of animals, the significance of the differences between the value observed on day 8 and the other values observed was tested by analysis of variance (ANOVA) and Tukey-Kramer's multiple range test. The weight percentage of 22:6n-3 and 18:1n-9 determined in trout acclimated in winter at 8°C (▲,❖) or in summer at 22°C (▼,◆) appears on the right-hand side of the figure for comparison. The difference between the two means was tested by the paired two-tailed Student's *t*-test. CA, cold acclimation. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.005$; $^{d}P < 0.001$.

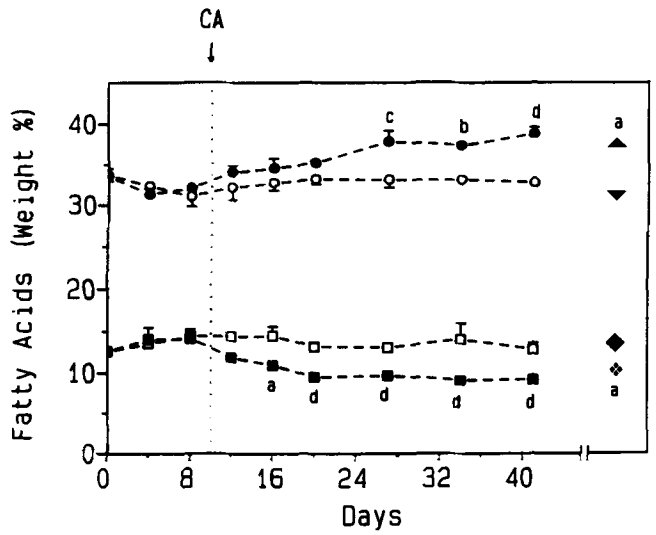

FIG. 2. Changes in the unsaturation index (UI) of fatty acids of plasma phospholipids during CA in juvenile trout. For details in the experimental design, see Materials and Methods. Values are means ± SEM, n = 5 (SEM within data points are not shown). For each group of animals, cold-acclimated (●) or summer-acclimated trout (○), the significance of the differences between the value observed on day 8 and the other values observed was tested by ANOVA and Tukey-Kramer's multiple range test. The UI determined in trout acclimated in winter at 8°C (▲) or in summer at 22°C (▼) appears on the right-hand side of the figure for comparison. The difference between these two means was tested by the paired two-tailed Student's *t*-test. $^{a}P < 0.005$; $^{b}P < 0.001$. See Figure 1 for abbreviations.

ferent PL molecular species and in their proportion of unsaturated FA (3–5). The changes in the FA composition of plasma PL that can be induced by thermal adaptation have not been investigated previously in poikilotherms. Our data show that adaptive compositional changes occur in response to natural changes in environmental water temperature. The present study also demonstrates that the changes in the FA composition of plasma PL observed during seasonal thermal adaptation can also be produced in juvenile trout by imposing controlled and rapid temperature changes in the laboratory. The changes that occur due to low temperature are consistent with the prior descriptions of CA effects in trout liver PL (11,15). The increase in the UI of plasma PL FA during CA, or during seasonal adaptation to low temperature in winter is primarily due to a very significant increase in the relative proportion of PUFA, e.g., 22:6n-3. CA results in a decrease in the quantity of 18:1n-9 in plasma PL as in hepatic PL (11,15). However, there is a decrease of 18:0 in plasma PL instead of palmitic acid (16:0) as it is observed in hepatic PL (11,15). A time course study revealed that the changes in plasma PL FA became significant after 10 d of CA. A comparison between the UI of fatty acids of 6°C-cold-acclimated and 8°C-winter-acclimated trout indicates that the process is complete after one month. This period has been previously shown to be adequate for thermal adaptation of various physiological functions in fish (16).

In fish, almost all plasma lipids are associated with lipoproteins (17), which, in turn, transport and distribute endogenous and exogenous lipids within the organism. In trout, high density lipoproteins (HDL) predominate in plasma lipoproteins (17–19). HDL lipids are rich in unsaturated FA of the linolenic acid (n-3) family (20). Changes in the composition of the fatty acyl chains of plasma total PL observed in trout during CA or low-temperature seasonal adaptation indicate that the FA composition of plasma lipoprotein PL does not remain constant during thermal adaptation. These modifications may be due to changes in the molecular species composition of PL classes and/or in the proportion of each PL in plasma lipoproteins. We have recently demonstrated in trout that the weight percentage of 22:6n-3, the U/S ratio and the UI of FA of HDL PL are inversely correlated with water temperature (12). The rapid restructuring of PL in the liver at low temperature and the later increase in the proportion of unsaturated FA due to the stimulation of hepatic desaturation activities (for a review, see Ref. 4) would result in the release of lipoproteins whose FA composition has been adapted to environmental changes. Thus, the plasma lipoproteins can rapidly supply lipids with the degree of unsaturation required for thermal adaptation.

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ARTICLE

Alteration of Chain Length Selectivity of a *Rhizopus delemar* Lipase Through Site-Directed Mutagenesis[1]

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The coding sequences of the *Rhizopus delemar* lipase and prolipase were altered by oligonucleotide-directed mutagenesis to introduce amino acid substitutions. The resulting mutant enzymes, synthesized by the bacterial host *Escherichia coli* BL21 (DE3), were tested for their ability to hydrolyze the triglycerides triolein (TO), tricaprylin (TC) and tributyrin (TB). Mutagenesis and lipase gene expression were carried out using plasmid vectors derived from previously described recombinant plasmids [Joerger and Haas (1993) *Lipids 28*, 81–88] by introduction of the origin of replication of bacteriophage f1. Substitution of threonine 83 (thr83), a residue thought to be involved in oxyanion binding, by alanine essentially eliminated lipolytic activity toward all substrates examined (TB, TO and TC). Replacement of thr83 with serine caused from two- to sevenfold reductions in the activity toward these substrates. Introduction of tryptophan (trp) at position 89, where such a residue is found in closely related fungal lipases, reduced the specific activity toward the three triglyceride substrates. For the mutagenesis of residues in the predicted acyl chain binding groove, mutagenic primers were designed to cause the replacement of a specific codon within the prolipase gene with codons for all other amino acids. Phenylalanine 95 (phe95), phe112, valine 206 (val206) and val209, were targeted. A phenotypic screen was successfully employed to identify cells producing prolipase with altered preference for olive oil, TC or TB. In assays involving equimolar mixtures of the three triglycerides, a prolipase with a phe95 → aspartate mutation showed an almost twofold increase in the relative activity toward TC. Substitution of trp for phe112 caused an almost threefold decrease in the relative preference for TC, but elevated relative TB hydrolysis. Replacement of val209 with trp resulted in an enzyme with a two- and fourfold enhanced preference for TC and TB, respectively.
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The fungus *Rhizopus delemar* [presently designated *R. oryzae* (1)] produces extracellular lipases that have been used in research for many years. One of these lipases has been purified and characterized (2), and a complementary DNA (cDNA) encoding this lipase has been cloned and its nucleotide sequence has been determined (3). This lipase, like its closely related counterpart produced by the fungus *Rhizomucor miehei* (4), exhibits a strong preference for the hydrolysis of ester bonds at the *sn*-1 and *sn*-3 positions of a triacylglycerol. As opposed to this pronounced positional selectivity, there is little selectivity for hydrolysis of substrates with different fatty acid moieties. In assays with triglyceride mixtures, tributyrin (TB) was the least preferred substrate. Triacylglycerols with longer chain fatty acids, both saturated and unsaturated, were hydrolyzed at only marginally different rates (5). In contrast, some lipases from *Candida cylindracea* exhibit little positional specificity (6), a lipase from *Geotrichum candidum* has a strong preference for the hydrolysis of *cis*-9 unsaturated fatty acids (6,7) and a lipase from *Penicillium camembertii* (Pc) hydrolyzes only mono- and diglycerides (8). The molecular bases for these selectivities are either still completely unknown or are just beginning to be revealed. Three-dimensional structures determined by X-ray crystallography are now available for several lipases (9–12), and continued refinement and analyses will provide new insights into the phenomenon of selectivity.

Rational mutagenesis provides another route for investigating the structure–function relationship of enzymes (13). Using this technique, it should be possible to gather useful information regarding the role of sequence and structure in determining the activity, selectivity and stability of these enzymes. In order to facilitate studies on the *R. delemar* (Rd) lipase, the cloned cDNA encoding this lipase was used to construct expression plasmids for the high-level production of the proenzyme and mature form of the lipase in the bacterium *Escherichia coli* (14).

We have now modified the expression plasmids to create plasmids that permit site-directed mutagenesis and expression to be carried out with the same vector. This system was used to create mutations in the lipase and prolipase genes and to express these altered genes. The gene products were then tested for their ability to hydrolyze TB, tricaprylin (TC) and triolein (TO).

MATERIALS AND METHODS

DNA manipulations. Plasmid DNA was isolated by an alkaline lysis procedure (15) followed by phenol/chloroform extraction. Single-stranded DNA was isolated from cul-

[1]Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: aa, Amino acid; ala, alanine; asp, aspartate; cDNA, complementary deoxyribonucleic acid; gly, glycine; Hl, *Humicola lanuginosa*; IPTG, isopropyl-β-D-thiogalactoside; leu, leucine; Pc, *Penicillium camembertii*; phe, phenylalanine; Rm, *Rhizomucor miehei*; Rd, *Rhizopus delemar*; ser, serine; TB, tributyrin; TC, tricaprylin; TE buffer, Tris/ethylenediaminetetraacetic acid buffer; thr, threonine; TO, triolein; Tris, tris(hydroxymethyl)aminomethane; trp, tryptophan; val, valine.

ture fluids by precipitation with 1/4 vol of 20% polyethylene glycol in 3.75 M ammonium acetate (NH_4OAc). The precipitate was resuspended in Tris/ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 8) and extracted repeatedly with phenol/chloroform. DNA was stored in TE buffer. Restriction enzyme digestions and ligations were performed as recommended by the suppliers. Plasmid DNA was introduced into *E. coli* cells either by transformation of $CaCl_2$-treated cells (16) or by electroporation using a Gene Pulser™ (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions. Sequencing of DNA was carried out by the method of Sanger *et al.* (17) using the Sequenase™ Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

Vector construction. The origin of replication of bacteriophage f1 was introduced into the lipase expression plasmid pET11-d-431 (14) in a procedure similar to that described by Richardson and Richardson (18). The f1 origin of replication was isolated from plasmid pTZ18R (purchased from Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as a 1060-bp *Sca*I-*Hind*III fragment. Plasmid pET11-d-431 was cleaved with restriction enzyme *Sca*I and the linearized plasmid incubated with *Hind*III under conditions favoring partial digestion. The resulting fragments were separated by agarose gel electrophoresis, and the band corresponding to a *Sca*I-*Hind*III fragment approximately 6 kbp in size was excised from the gel, extracted and purified. This fragment was ligated to the f1 ori-containing *Sca*I-*Hind*III fragment from pTZ18R to yield a plasmid, pET11-d-f1-431, that retained the features required for lipase gene expression and also allowed production of single-stranded circular DNA for use in site-directed mutagenesis.

Under inducing conditions, cells harboring pET11-d-f1-431 produce inactive lipase that has to be refolded into active enzyme. Direct production of active mature Rd lipase in *E. coli* is not possible due to the toxic nature of this enzyme. However, a plasmid allowing expression of active prolipase in *E. coli* BL21 (DE3) (19) was constructed. To achieve this, the *Eco*RI-*Xba*I fragment from pET11-d-f1-431, which contains the gene for mature lipase, was replaced with an *Eco*RI-*Xba*I fragment encoding prolipase. This fragment was isolated from plasmid pTM-N-1231, which consists of vector pTM-N (20) and the Rd prolipase gene fused to DNA encoding the OmpA signal peptide. The new plasmid, pET11-d-f1-1231s, was used for isolation of single-stranded DNA and for production of active prolipase.

Plasmids for the production of mature lipases carrying the amino acid substitutions found in previously characterized prolipases were constructed by replacement of the 600-bp *Kpn*I-*Eco*RI fragment in pET11-d-f1-431 with the corresponding fragments from the pET11-d-f1-1231s mutant plasmids. These pET11-d-f1-431 derivatives were used for the production of mature mutant lipases in inactive forms as inclusion bodies. Active enzyme was obtained after refolding as described previously (14).

Production of single-stranded DNA from pET11-d-f1-431 and pET11-d-f1-1231s. Single-stranded DNA was obtained by infecting plasmid-containing *E. coli* JM101 cells with helper phage M13KO7 as described by Viera and Messing (21). Packaged single-stranded DNA was precipitated with PEG/NH_4OAc and purified by repeated phenol/chloroform extractions. For the production of single-stranded DNA for use in mutagenesis experiments according to the methods of Kunkel (22), plasmids were first introduced into *E. coli* strain CJ236. Uracil-containing single-stranded DNA was prepared from the culture supernatant of M13KO7-infected cells as indicated above.

Oligonucleotide-directed mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel (22) using the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad Laboratories). Mutagenic primers were obtained from the Department of Chemistry, University of Pennsylvania (Philadelphia, PA). The nucleotide sequences of the primers used are listed in Table 1. Primer-mediated nucleotide sequence changes affecting restriction enzyme recognition sites were so chosen as not to alter the amino acid sequence of the lipases.

TABLE 1

Mutagenic Primers

Primer nucleotide sequence[a]	Amino acid change introduced[b]	Other changes introduced[c]
5′TCCGTGGTGCTAACTCCTTC3′	thr83- > ala	removal of *Kpn*I site at pos. 658
5′TCCGTGGTTCCAACTCCTTC3′	thr83- > ser	removal of *Kpn*I site at pos. 658
5′TCAGAAGTTGGATCACTGACATCGTCTTC3′	ala89- > trp	removal of *Eco*RV site at pos. 688
5′CACTGATATTGTCNNXAACTTTTCT3′	phe95- > aa	removal of *Eco*RV site at pos. 688
5′AGTTCATGCCGGCNNXCTTTCCTCT3′	phe112- aa	introduction of *Nae*I site at pos. 742
5′GAGAGATATTNNXCCTCACN NXCCTCCTCAA3′	val206- aa, val209- aa	removal of *Eco*RV site at pos. 1024

[a]During primer synthesis, an equimolar amount of A, C, G and T was used at the positions indicated by the letter N. Only equimolar amounts of C and G were used at the positions indicated by letter X.

[b]Amino acids are numbered as in Reference 3; aa indicates that any one of the twenty possible amino acids could be present after mutagenesis. Abbreviations: thr, threonine; ala, alanine; ser, serine; trp, tryptophan; phe, phenylalanine.

[c]The pos. numbers are as in Reference 3 and denote the position of the first nucleotide of the restriction enzyme recognition site.

Mutant screening. Mutated versions of pET11-d-f1-431 were identified initially by acquisition or loss of a restriction enzyme site (see Table 1). DNA sequencing was then used to confirm the presence of the desired base changes. A phenotypic screening for cells carrying mutated versions of pET11-d-f1-1231s consisted of replica-plating cells onto solid LB-agar medium (rhodamine B medium) containing dispersed triglyceride (25 mL/L), ampicillin (100 μg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) and rhodamine B (0.0005%) (3). Fatty acids produced by lipase activity cause the rhodamine B to acquire a bright pink color and to fluoresce under ultraviolet light. Cells acquire a similar coloration and become surrounded by halos. Lipase negative cells remained cream-colored. Plasmid DNA was isolated from cells producing mutant lipases of interest for this study, and the nucleotide sequence of the region expected to carry a mutation was determined.

Induction of lipase gene expression. Escherichia coli BL21 (DE3) harboring recombinant plasmids were grown on solid LB medium (23) containing ampicillin (100 μg/mL). Cells containing pET11-d-f1-431 or its mutagenized derivatives were cultured in liquid medium and induced for lipase expression by the addition of IPTG to a final concentration of 1 mM as previously described (14). Cells containing pET11-d-f1-1231s or its mutant forms were induced in liquid culture (2 to 10 mL of LB medium containing ampicillin) by the addition of IPTG to a final concentration of 0.2 mM when the optical density (at 600 nm) of the culture reached 0.7 to 1.0. Cells were harvested 3 to 4 h after the addition of inducer.

Preparation of mature and prolipase samples. Wild-type and mutant versions of mature lipase were refolded and purified, and their purity and specific activities were determined as previously described (14). Briefly, the lipase inclusion bodies were solubilized in 8 M urea, and refolding was initiated by dilution. The refolded lipase preparation was purified to homogeneity by affinity and ion-exchange chromatography. Purity was assessed visually by examination of acrylamide electrophoresis gels stained with Coomassie Blue.

Cells induced for prolipase production were resuspended in 20 mM sodium phosphate buffer, pH 6.5, and sonicated. The lysate was used directly in lipase activity assays.

Determination of lipase activity. Activity determinations were carried out titrametrically as described previously (14) using a VIT 90 Video titrator (Radiometer, Copenhagen, Denmark) and 0.1 N NaOH as titrant. The assays were conducted at 25°C at pH of 7.5. The reaction mixture consisted of 200 mM olive oil, TC or TB (Sigma, St. Louis, MO) in 4.2% aqueous gum arabic solution and 14 mM $CaCl_2$. Lipase activity was calculated from the maximum rate of titrant addition using a lipase titrametric assay program (Cichowicz, D.J., *et al.,* unpublished). A unit (U) of activity released one μmole of fatty acid per minute.

Substrate selectivity was determined in an assay containing 500 μL of 50 mM sodium phosphate, pH 7.5, and 200 μmoles each of TO, TC and TB. The reactions were started by the addition of purified mature enzyme or prolipase-containing lysates. Samples to which only buffer or heat-inactivated cell sonicate had been added served as controls. The tubes were incubated at 30°C on an orbital shaker that was rotated at 300 rpm. After two hours, 20 μmoles of palmitic acid in 50 μL pyridine was added to the tubes to serve as internal standard, the content of the tubes was thoroughly emulsified, and 10 μL was quickly withdrawn to another tube. Then, 100 μL of dry pyridine was added, followed by 400 μL of *bis*(trimethylsilyl)trifluoroacetamide (Sigma). The tubes were incubated for 15 min in a boiling waterbath (24), cooled to room temperature, and hexane was added to obtain a solution appropriately diluted for gas chromatography. The samples were injected on-column into a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph containing a 15-m nonpolar high-temperature column (DB1-HT) with an inner diameter of 0.32 mm and a film thickness of 0.1 μm (J&W Scientific, Folsom, CA). The helium flow rate was 5.5 mL per min. Detection was by flame ionization. The oven temperature was maintained at 40°C for 0.5 min, then increased to 100°C at a rate of 20°C per min, and then increased to 120°C at a rate of 2°C per min. The temperature was finally raised to 350°C at a rate of 20°C per min. This triphasic temperature profile was necessary to separate TB from monocaprylin. Data collection and processing was done on a Hewlett-Packard 3396 SeriesII Integrator.

RESULTS AND DISCUSSION

Mutagenesis experiments. The system for the expression of the Rd prolipase and mature lipase genes in *E. coli* described previously (14) allowed the synthesis and subsequent purification of relatively large amounts of these lipases. The modification of the expression vectors to include the bacteriophage f1 origin of replication was undertaken to produce vectors that can be used to bring about mutagenesis and expression without cloning in two different vectors. This approach had been proven successful previously in studies of the gene for the transcription termination factor, rho (18).

In initial experiments, threonine (thr) 83 was replaced with either alanine (ala) or serine (ser). Thr83 occupies the same position in the Rd lipase as ser82 in the *Rhizomucor miehei* (Rm) lipase (4). This residue has been implicated in oxyanion binding during hydrolysis of triglycerides by the Rm lipase (9,10). Cells harboring mutagenized plasmids were induced to produce inactive lipase in the form of inclusion bodies. The mutant lipases were then refolded, purified and characterized (14). Replacement of thr83 with ala caused a decrease in the specific activity by almost three orders of magnitude (Table 2). A similar loss of activity was observed when a corresponding ser residue was replaced by glycine (gly) in the mono- and diglyceride lipase from Pc (25). These reductions in activity are not unexpected because the loss of anion stabilization would be expected to profoundly reduce the activity of the enzyme. Neither gly nor ala can supply a hydroxyl group to attain this stabilization. On the other hand, replacement of thr83 with ser resulted in a lipase whose specific activity on various substrates was between one-fifth and one-half that of the wild type (Table 2). Presumably, the ser hydroxyl can partially substitute for the thr hydroxyl, but

TABLE 2

Titrametric Analysis of Lipolytic Activity of Refolded and Purified Recombinant Lipases

Source[a]	Mutation[b]	Specific activity[c]		
		Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-431	wild type	3149	8539	2944
pET11-d-f1-431-T83A	thr83- > ala	4	7	1
pET11-d-f1-431-T83S	thr83- > ser	1664	3350	442
pET11-d-f1-431-A89W	ala89- > trp	2106	6628	1702

[a]Expression plasmid present in *Escherichia coli* BL21 (DE3).

[b]Amino acids numbered as in Reference 3. See Table 1 for abbreviations.

[c]Micromoles fatty acid released per min per mg of protein. The values are averages of two experimental determinations.

the difference in the positioning of the side chains of the two amino acids is significant enough to reduce the specific activity of the ser variant.

Residues 86 to 92 in the Rd lipase are predicted to form a "lid" that would cover the active site of the lipase, as was shown by crystallographic studies of the related Rm lipase (9,10). It has been postulated, and demonstrated using inhibitors (9,10), that this lid is displaced when substrate analogues bind to the active site. This displacement exposes the active site and increases the potential lipase-substrate interface by enlarging the hydrophobic surface area of the enzyme. These features are thought to underlie the requirement, demonstrated by all lipases, that an interface be present before activity is manifested. Ala89 occupies a position in the Rd lipase where tryptophan (trp) is found in the lipases from *R. miehei, Humicola lanuginosa* (Hl) and Pc (25; Derewenda, Z.S., personal communication). It was postulated that this trp might play a role in the interfacial activation process (9). Recently, Clausen *et al.* (26) reported that in the Rm lipase, replacement of the trp did not abolish interfacial activation, but that substrate penetration was changed. By replacing ala89 in the Rd lipase with trp, we attempted to obtain more insight into the role of hydrophobic residues in the lids of these fungal lipases, especially as it relates to enzyme–substrate interaction. We found that replacement of ala89 with trp caused a 22 to 42% reduction in the specific activity relative to the corresponding activity of the purified wild-type lipase (Table 2). However, compared to the wild-type enzyme there was no profound change in the relative activity of this modified enzyme toward the various substrates tested (Tables 2, 3; Figs. 2, 3). Further studies, such as determination of the interfacial activation curve of the wild-type and mutant enzymes, and elucidation of the three-dimensional structures of these proteins will be required in order to precisely identify the roles of specific amino acids in the lid of the enzyme.

The plasmid system described above for the mutagenesis and expression of the thr and ala mutants has the advantage of supplying relatively large quantities of pure lipase, but the disadvantage of requiring isolation of the inactive lipase as inclusion bodies with subsequent refolding to produce active enzyme. Despite many attempts to find an expression system that would allow the production of active, mature lipase, no system could be developed that was able to overcome the toxic effect of this enzyme on the bacterial host (14). Thus, a plasmid that allows the production of active prolipase, which appears to be less toxic, was constructed for the experiments with mutagenic primers containing a randomized codon (Table 1). Our goal was to determine whether or not it was possible to alter the substrate specificity of the Rd lipase by replacement of specific amino acid residues. The residues chosen [phenylalanine (phe) 95, phe112, valine (val) 206, val 209], are well conserved in the Rd, Rm, Hl and Pc enzymes and are predicted to reside in a groove that might accommodate the acyl chain of a substrate glyceride bound in the active site (9,10) (Fig. 1).

TABLE 3

Hydrolysis of an Equimolar Mixture of Triolein, Tricaprylin and Tributyrin by Refolded, Purified Recombinant Mature Lipases

Source	μg of lipase in assay	% Hydrolyzed[a]		
		Triolein	Tricaprylin	Tributyrin
pET11-d-f1-431	2.5	80.1	83.9	42.4
pET11-d-f1-431-T83A	435.0	21.8	24.2	5.7
pET11-d-f1-431-T83S	2.7	58.0	56.4	10.2
pET11-d-f1-431-A89W	2.5	60.4	72.6	17.4

[a]Percent hydrolyzed = $(TG_C - TG_H)/(TG_C) \times 100$ where TG_C equals the amount of triglyceride present in enzyme-free reaction and TG_H is the amount of triglyceride remaining after incubation with the lipase.

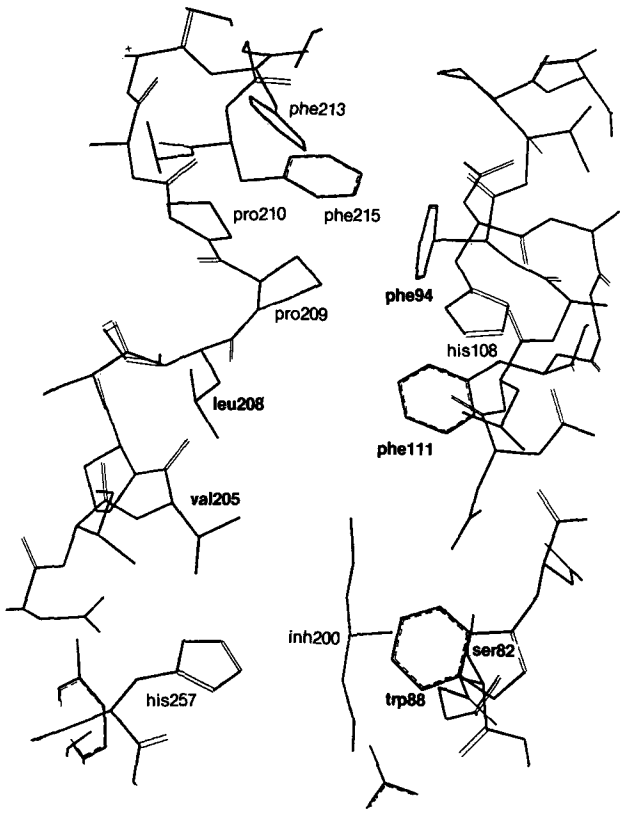

FIG. 1. Structure of a part of the *Rhizomucor miehei* (Rm) lipase with the active site inhibitor (INH200) *n*-hexylphosphonate ethylester (9). The oxyanion-binding ser82 as well as residues lining the fatty acid-binding groove are shown. The corresponding residues in the *Rhizopus delemar* lipase are thr83 in place of Rm (ser82), phe95 (phe94), his109 (his108), phe112 (phe111), val206 (val205), val209 (leu208), pro210 (pro209), pro211 (pro210), phe214 (phe213), phe216 (phe215), his257 (his257). Crystallographic coordinates kindly provided by Z. Derewenda. Abbreviations: ser, serine; thr, threonine; phe, phenylalanine; val, valine; pro, proline; his, histidine.

To scan large numbers of mutants, the phenotypic lipase assay of Kouker and Jaeger (27) was employed. In this procedure, colonies arising after mutagenesis were replicated onto rhodamine medium (see Materials and Methods section). Thus colonies could be identified that differed from the wild type in their capacity to hydrolize the components of olive oil or TC. This phenotype was ascertained to be stable by retesting on the rhodamine B media. The majority of colonies arising after mutagenesis had an appearance on rhodamine B media that was indistinguishable from that of wild-type colonies. This is due in part to mutagenesis efficiencies of less than 100%. However, the wild-type phenotype predominated even among colonies that contained mutated plasmids as indicated by the occurrence of a mutagenic primer-induced restriction enzyme susceptibility. This suggests that, despite the conserved character of the residues subjected to mutagenesis, a number of replacements are tolerated without a major change in the lipolytic phenotype. The nature of these neutral substitutions is not known because no sequence determinations were made. A second group of mutants exhibited a lipase-negative phenotype. Presumably, some of the substituting amino acids have side chains that prevent either proper folding of the enzyme or the conformational changes required for interfacial activation, substrate binding or catalysis.

Hydrolysis of olive oil, TO, TC and TB. The activities of mutant lipases were tested titrametrically against the individual substrates. For the pure wild-type and mutant mature enzymes, specific activities could be assigned for each substrate (Table 2) and a profile of the relative activities, with the activity against olive oil set at 100%, was established (Fig. 2). The reduction in the specific activity as a result of the replacement of thr83 by ser or ala has been noted previously. In addition to this reduction in activity, these mutant enzymes display an alteration in their substrate preferences, with the activity toward TB, relative to that toward TO, being reduced roughly threefold compared with the wild-type enzyme (Fig. 2). The titrametric assay using individual substrates allowed the relatively rapid screening of potential mutant lipases and provided information concerning the activities of the enzymes. However, to overcome differences in the emulsion properties of TB, TC and olive oil, experiments were also conducted that presented the enzymes with mixtures of three substrates. Activity was determined by measuring the disappearance of each substrate during incubation with the enzymes. TO was used in place of olive oil in these assays to simplify the gas-chromatographic analyses. As with the wild-type enzyme, purified mutant lipases had similar activities toward TC and TO (Table 3). However, compared to the wild-type enzyme, the thr83 and ala89 mutant lipases showed a reduction in their relative activities toward TB (Table 3, Fig. 3).

The lipolytic activities, toward individual triglyceride substrates, of lysates containing wild-type or mutant prolipases are listed in Table 4. Since these data were collected using impure enzyme preparations, the activities are listed in units per mL of cell lysate, and absolute activity values cannot be compared with one another. Nevertheless, the relative activities can be compared (Fig. 4A). As seen with the mature wild-type lipase (Table 2, Fig. 2),

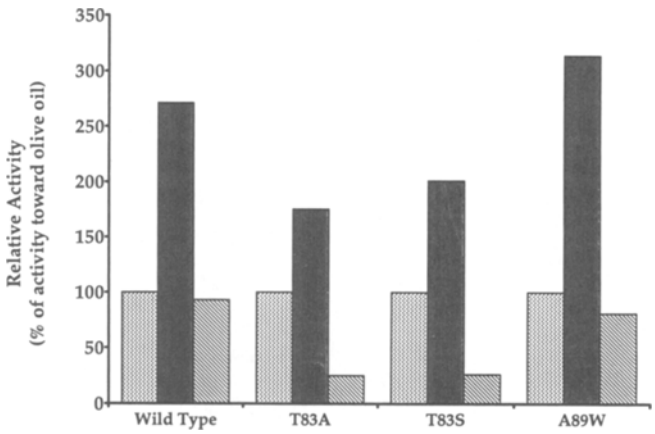

FIG. 2. Relative activities calculated from the results of the titrametric assays on olive oil (dotted bars), tricaprylin (dark bars), and tributyrin (hatched bars), listed in Table 2. The activity on olive oil was set at 100%.

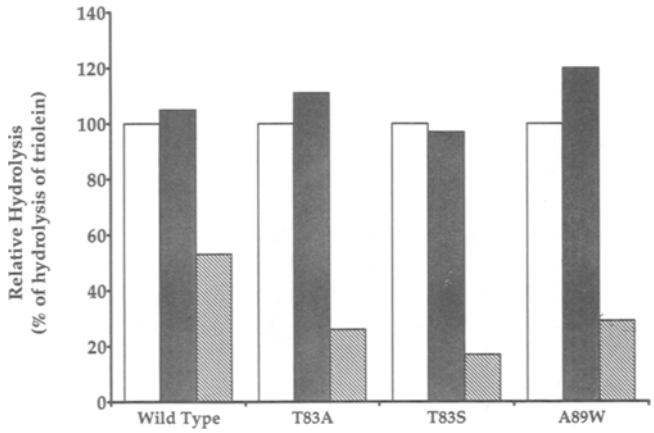

FIG. 3. Relative activities calculated from the results, listed in Table 3, of hydrolysis experiments with substrate mixtures composed of triolein (light bars), tricaprylin (dark bars) and tributyrin (hatched bars). The percent hydrolysis of triolein was set at 100%.

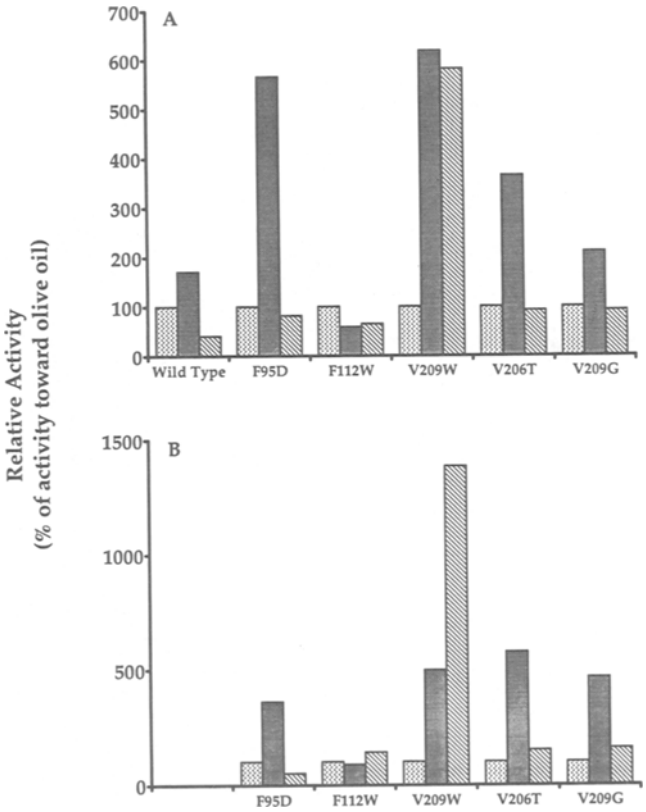

FIG. 4. The relative lipolytic activities of prolipase (A) and mature lipase (B) mutants against the single substrates olive oil (dotted bars), tricaprylin (dark bars) and tributyrin (hatched bars). Activity on olive oil was set at 100%. Data from Tables 4 and 6, respectively.

the wild-type prolipase is most active with TC as substrate and least active against TB. This was confirmed by incubation of the enzymes in the simultaneous presence of the three substrates (Table 5, Fig. 5A). However, there was only a small difference between the hydrolysis of TO and TC in this case as opposed to the experiments using single substrates (Table 4).

The prolipase expressed from pET11-d-f1-1231s-F95D exhibits more than a threefold higher activity toward TC than does the wild enzyme in the single substrate titration assay (Table 4, Fig. 4A), and an almost twofold higher activity in the mixed substrate assay (Table 5, Fig. 5A). Whether this is caused by a higher hydrolytic activity toward TC or a reduced activity toward TO cannot be determined with these impure preparations.

Replacement of phe112 by trp greatly effected the substrate preference of the prolipase. In contrast to the wild type, the lysate from a strain harboring pET11-d-f1-1231s-F112W exhibited its lowest activity toward TC (Tables 4, 5). Relative to the wild-type enzyme, this mutant enzyme hydrolyzed TB about 50% better, with the ratio of TO activity to TB activity being 1.6, compared to 2.4 for the wild type (Table 4, Fig. 4A). Against mixed substrates, TC was again least preferred, while TB was hydrolyzed to a large extent (Table 5, Fig. 5A). Thus the mutagenic replacement of a large side chain by an even bulkier one reduced the relative activity toward triglycerides with medium-chain fatty acids and increased activity toward those with short chains.

Mutant plasmid pET11-d-f1-1231s-V209W encodes a prolipase with a substitution of val209 by a trp residue. *Escherichia coli* cells bearing this plasmid produced a prolipase with highest activity on TB in the mixed substrate

TABLE 4

Lipolytic Activity in Lysates of Prolipase-Producing *Escherichia coli* BL21 (DE3)

Source	Mutation	Activity (U/mL)[a]		
		Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-1231s	wild type	158	270	65
pET11-d-f1-1231s-F95D	phe95- > asp	35	198	29
pET11-d-f1-1231s-F112W	phe112- > trp	100	59	65
pET11-d-f1-1231s-V209W	val209- > trp	16	99	93
pET11-d-f1-1231s-V206T	val206- > thr	64	234	41
pET11-d-f1-1231s-V209G	val209- > gly	54	114	50

[a]Data presentation as in Table 2, but activities expressed as U per mL of lysate from induced cells; asp, aspartate; val, valine; gly, glycine; see Table 1 for other abbreviations.

TABLE 5

Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin and Tributyrin by Prolipase in Cell Lysates

Source	Mutation	% Hydrolysis[a]		
		Triolein	Tricaprylin	Tributyrin
pET11-d-f1-1231s	wild type	66.8	75.8	39.0
pET11-d-f1-1231s-F95D	phe95- > asp	36.9	67.7	34.1
pET11-d-f1-1231s-F112W	phe112- > trp	18.4	7.2	67.7
pET11-d-f1-1231s-V209W	val209- > trp	18.2	35.4	72.0
pET11-d-f1-1231s-V206T	val206- > thr	66.1	72.4	43.0
pET11-d-f1-1231s-V209G	val209- > gly	54.8	60.0	60.0

[a]Calculated by the method defined in Table 3. Abbreviations as in Tables 1 and 4.

assay (Table 5, Fig. 5A) and toward TC and TB in the single substrate assay (Table 4, Fig. 4A). Apparently, this enzyme has a relatively low activity on TO, but as before, it will only be possible to verify this through purification of the prolipase. As with the mutant prolipase (F112W) described above, the change in substrate preference of the V209W mutant prolipase toward the substrate with the shortest acyl chains, TB, was caused by the replacement of a hydrophobic amino acid, val, with a bulky residue, trp.

The replacement of val206 with thr, and the replacement of val209 with gly, caused an increase in the relative activity of the prolipases toward TC in the single substrate assay (Fig. 4A). However, in the mixed substrate assay, the V206T mutant prolipase hydrolyzed the three substrates in a manner similar to that of the wild-type enzyme and the V209G mutant prolipase hydrolyzed each of the three substrates almost equally (Fig. 5A).

The production of mutant lipases as active proenzymes is an efficient way to rapidly screen for enzymes with altered properties. The presence of the propeptide and the need to use crude preparations, however, raises the question as to whether the properties observed are also those that would be observed with the corresponding mature enzymes. In order to address this question, the mutations carried by the prolipase genes were introduced into the gene for mature lipase by replacement of a *Kpn*I-*Eco*RI fragment from this gene with the corresponding fragments from the mutated prolipase genes. The resulting mutant lipase genes were expressed, and active mutant lipase enzymes were obtained after refolding of the inactive enzymes from inclusion bodies. Specific activities were obtained for the partially purified lipase preparations for the single substrates olive oil, TC and TB (Table 6). The relative activities (activity toward olive oil equals 100%) are shown in Figure 4B. Comparison of the data in Figure 4A and 4B indicates that the crude prolipase preparations and the partially purified refolded mature lipase samples behave similarly. Differences in the relative activities toward TC, mainly by the V209W mutant, however, could be

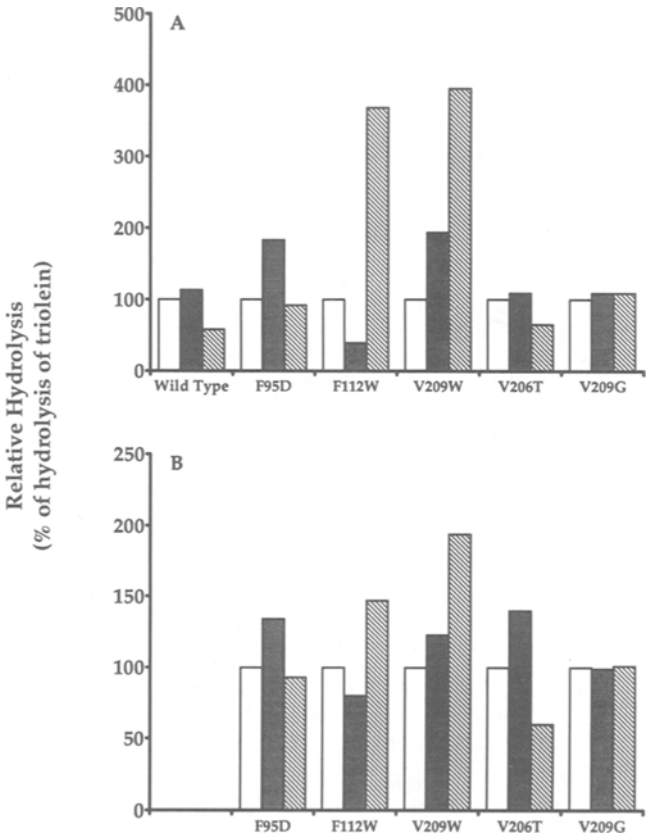

FIG. 5. The relative activities of wild-type and mutant lipases against mixtures of the substrates triolein (light bars), tricaprylin (dark bars) and tributyrin (hatched bars); (A) prolipase, data taken from Table 5; (B) mature lipase, data taken from Table 7. The activity toward triolein was set at 100%.

TABLE 6

Titrametric Analysis of Lipolytic Activity of Partially Purified, Refolded Recombinant Mature Lipases

Source[a]	Specific activity[b]		
	Olive oil	Tricaprylin	Tributyrin
pET11-d-f1-431-F95D	821	2978	404
pET11-d-f1-431-F112W	1279	1127	1800
pET11-d-f1-431-V209W	281	1398	3896
pET11-d-f1-431-V206T	187	1079	281
pET11-d-f1-431-V209G	187	466	296

[a]Plasmid present in *Escherichia coli* BL21 (DE3).

[b]Micromoles fatty acid released per min per mg of protein. The values are averages of two experimental determinations.

TABLE 7

Hydrolysis of Equimolar Mixtures of Triolein, Tricaprylin and Tributyrin by Partially Purified, Refolded Mutant Mature Lipases

Source	% Hydrolysis[a]		
	Triolein	Tricaprylin	Tributyrin
pET11-d-f1-431-F95D	59.3	79.5	55.3
pET11-d-f1-431-F112W	40.3	32.1	58.7
pET11-d-f1-431-V209W	34.3	42.5	66.4
pET11-d-f1-431-V206T	15.6	21.3	8.9
pET11-d-f1-431-V209G	47.1	46.7	47.6

[a]Values are average from two assays; 5 μg of protein was added to each reaction tube.

noted. The reasons for these differences remain to be elucidated.

The refolded mutant lipase samples were also tested in mixed substrate assays (Table 7). As seen in the single substrate assay, the relative hydrolysis data for the mutant prolipases and the mature mutant lipases closely resembled each other (Fig. 5).

Overall, the phenotypes observed so far indicate that it is possible to manipulate the substrate specificity of the Rd lipase through site-directed mutagenesis. Additional mutagenesis experiments, involving such targets as phe214 and phe216 (see Fig. 1), or deletions or insertions near residues that might interact with the acyl chain of triglycerides, should increase the spectrum of mutants. The combining of different mutations into one mutant lipase might yield enzymes with even higher substrate preferences. The determination of the three-dimensional structure of the Rd lipase (28), should allow the identification of additional mutagenesis targets and possibly provide explanations for the phenotypes caused by the mutations studied here.

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Phosphorylation of Lipids in Rat Primary Glial Cells and Immortalized Astrocytes (DITNC)

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Phosphatidylinositol 4,5-bisphosphate, a substrate in the signal transduction pathway involving phospholipase C, is synthesized in cell membranes by phosphorylation of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PI-4-P). Incubation with [γ-^{32}P]adenosine triphosphate of membranes isolated from primary glial cells in culture and from an immortalized astrocyte cell line (DITNC) and subsequent separation of the lipids by high-performance thin-layer chromatography revealed a number of labeled lipid bands. Further analysis of the deacylated products by high-performance liquid chromatography indicated the presence of two PI-4-P bands and a band corresponding to lysoPIP (PIP, PI phosphate). The two PI-4-P bands were more prominent in the samples from astrocyte cell membranes than from synaptic plasma membranes and plasma membranes. Analysis of the fatty acid composition of PI by gas–liquid chromatography revealed that both 20:3n-9 and 20:4n-6 were present in PI of cultured cell membranes, whereas the brain membranes contained exclusively 20:4n-6. The two PI-4-P bands in these membranes can be attributed to the presence of different molecular species of PI. Assignment of the fraction corresponding to lysoPIP was consistent with the observation that an increase in label in this lipid band occurred upon incubation of DITNC cell supernatant with lysoPI (1-stearoyl PI). This suggests that endogenous lysoPI present in the cell membranes can be phosphorylated to form lysoPIP.

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Although phosphoinositides (phosphatidylinositol, PI; phosphatidylinositol-4-phosphate, PI-4-P; and phosphatidylinositol 4,5-bisphosphate, PI-4,5-P_2) comprise only a small portion of cell membrane lipids, they play an important role in the cell signaling cascade (1,2). In particular, PI-4,5-P_2 serves as substrate for phospholipase C, which is stimulated by receptor agonists to yield two second messengers, namely diacylglycerol (DG) and inositol trisphosphate (IP_3) (2). The PI-4,5-P_2 pool, which is depleted upon cell stimulation, can in turn be replenished by sequential phosphorylation of PI catalyzed by PI- and PIP-kinases (PIP, PI phosphate) (3,4). In most instances, the second messengers generated in cells due to stimulated hydrolysis of PI-4,5-P_2 are rapidly removed, e.g., IP_3 *via* IP_3-kinase and phosphatase and DG *via* DG-kinase (1,2). Although different isoforms of DG-kinases are present in brain cytosol (5,6), some of these isoforms may associate with membranes through a calcium-mediated translocation process (6). Despite the important role of the lipid kinases in cell signaling mechanisms, factors modulating their activities have remained relatively unexplored.

In previous studies, incubation of brain subcellular membrane fractions with [γ-^{32}P]adenosine triphosphate (ATP) has been shown to result in the labeling of three major lipid classes, namely phosphatidic acid (PA), PI-4-P and PI-4,5-P_2 (7,8). However, the results from our recent experiments with membranes from cultured cells have indicated the presence of more than three labeled lipid bands after separation by high-performance thin-layer chromatography (HPTLC). There is evidence that some transformed cells can phosphorylate PI to yield phosphatidylinositol-3-phosphate (PI-3-P) and phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) (9–11). Therefore, a more detailed analysis of the lipids formed upon incubation of cell membranes with [^{32}P]ATP seemed warranted. The major objective for the present study was to examine lipid phosphorylation in primary glial cells as well as in an immortalized astrocyte cell line (DITNC) (12).

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear Research Products, Inc. (Boston, MA). [^{3}H]Inositol-1 monophosphate, inositol-1,4-bisphosphate and inositol-1,4,5-trisphosphate (1.0 Ci/mmol) were purchased from American RadioChemicals, Inc. (St. Louis, MO). ATP, polyPI, PA, lysoPA, PI-4-P and lysoPI were from Sigma Chemical Co. (St. Louis, MO). Whatman HP-K high-performance silica gel plates (10 × 10 cm, 200 μ thick) were obtained from Fisher Scientific Co. (St. Louis, MO). Sprague-Dawley rats were purchased from Taconic Farm (Germantown, NY). Partisil 10 SAX high-performance liquid chromatography (HPLC) analytical column (10 μm, 25 cm) with a Solvecon pre-column (25 cm) was from Whatman International Ltd. (Maidstone, England). BioSafe-NA scintillation fluid was from Research Product International Corp. (Mount Prospect, IL). UniverSol scintillation fluid was from ICN Radiochemicals (Covina, CA). Cell culture reagents were from Cell and Immunobiology Core Facilities (University of Missouri, Columbia, MO). Kodak X-OMAT-AR imaging films were from Eastman Kodak Company (Rochester, NY). An immortalized astrocyte cell line (DITNC) was kindly provided by Dr. C.F. Deschepper (Clinical Research Institute of Montreal, Montreal, Canada) (12). This cell

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Abbreviations: ATP, adenosine triphosphate; DG, diacylglycerol; DITNC, immortalized astrocytes from rat diencephalon; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethyleneglycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; G-3-P, glycerol-3-phosphate; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; IP, inositol monophosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; PI-3-P, phosphatidylinositol-3-phosphate; PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PI-4-P, phosphatidylinositol-4-phosphate; PI-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PIP, phosphatidylinositol phosphate; PM, plasma membranes; SPM, synaptic plasma membranes.

line was originally derived from rat diencephalon, and exhibits many of the characteristics of the type 1 astrocytes (12).

Cell culture and isolation of cell membranes. DITNC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin, 1% streptomycin and 1% fungizone. The primary glial cell culture was prepared from cerebral hemispheres of new-born Sprague-Dawley rats following the procedure described by Murphy (13) with minor modifications. Briefly, brains were minced and dissociated in a medium containing DMEM with 0.25% trypsin, and the mixture was incubated at 37°C for 20 min. After dissociation, cells were collected by centrifugation at 200 × *g* for 10 min and resuspended in the same medium as for DITNC cells. Cells were plated on poly-L-lysine-coated dishes and incubated at 37°C in a Steri-Cult 200 incubator (Forma Scientific, Marietta, OH) under a humidified atmosphere of 95% air and 5% CO_2. Culture medium was changed twice weekly, and cells were used for experiments after 2–4 passages. Microscopic examination of the primary glial cultures indicated the presence of astrocytes as well as a small proportion of microglial cells.

Cells were harvested by brief exposure to trypsin followed by neutralization, centrifugation and resuspension in buffer A containing 10 mM Tris-HCl (pH 7.4) with 10 mM $MgCl_2$ and 1 mM ethyleneglycol *bis*(β-aminoethyl ether (EGTA) (14). Cells were disrupted by sonication (5 times for 5 s each and with a 15-s interval between each burst). The broken cell suspension was centrifuged at 105,000 × *g* for 5 min to obtain membrane and supernatant fractions.

Synaptic plasma membranes (SPM) and somal plasma membranes (PM) were isolated from rat cerebrum by differential speed and sucrose gradient centrifugation according to the procedure described by Sun *et al.* (15). Membranes were suspended in buffer A (14).

Incubation of membrane fractions with [γ-^{32}P]ATP. The procedure used for incubation of membrane fractions with [γ-^{32}P]ATP was similar to that described by Stubbs *et al.* (7). In a typical incubation, 20 μg of membrane protein in 25 μL of buffer A was preincubated at 30°C for 5 min and then incubated with [γ-^{32}P]ATP (3 μCi in 7.5 μM, NEN) at 30°C for 60 s. The reaction was terminated by adding 3 mL of chloroform/methanol/12N HCl (2:1:0.025, by vol). The lipid extract was made acidic by adding 0.6 mL of 0.6N HCl to form a two-phase system. After phase separation, the lower organic phase was collected and washed twice with 1.5 mL of chloroform/methanol/0.6N HCl (3:48:47, by vol). The organic phase was dried under nitrogen and applied to an HPTLC plate that had previously been impregnated with a solution containing 1% potassium oxalate and 2 mM EGTA (diluted with methanol 3:2, vol/vol). In some instances, appropriate standards were added to the samples to aid in visualizing the fractions. The HPTLC plates were developed in a solvent system containing chloroform/methanol/acetone/ammonium hydroxide (35:20:5:5, by vol). After the first development, plates were dried and then developed again in the same direction using chloroform/methanol/ammonium hydroxide/water (36:28:2:6, by vol). After development, HPTLC plates were exposed to Kodak X-OMAT film for various periods of time to identify and quantify the fractions. Lipid fractions on HPTLC plates were visualized either by exposure to iodine vapors or by charring upon spraying with a copper acetate/phosphoric acid reagent (3 g copper acetate in 100 mL of 8% phosphoric acid). Bands were scraped into scintillation vials for measurement of radioactivity. In some instances, total lipid extracts or individual lipid classes recovered after one-dimensional HPTLC were further separated by two-dimensional HPTLC, as described by Sun (16).

In experiments that were designed to further analyze the lipid bands by HPLC, various amounts of membrane proteins and [γ-^{32}P]ATP were used so that enough labeled materials were obtained for HPLC analysis. In some experiments, exogenous lipids such as PA, PI, lysoPI and PIP were added to the incubation system to test their effects on the lipid kinases.

Deacylation of lipids and separation by HPLC. To further analyze the lipid bands by HPLC, it was necessary to deacylate the glycerolipids. The procedure used was similar to that described by Ascoli *et al.* (17) and Pignataro and Ascoli (18) with minor modifications. Briefly, individual adsorbent bands scraped from the HPTLC plates were transferred into test tubes, and the lipids were extracted with chloroform/methanol/0.2N HCl (5:10:4, by vol). A two-phase system was obtained by adding chloroform and water. The lower phase was neutralized with 60 μL of 1N ammonium hydroxide before drying under nitrogen. The dried samples were dissolved in 50 μL of chloroform and 75 μL of methanol and then saponified in 125 μL of 0.2N NaOH in methanol. After reaction at 25°C for 20 min, 200 μL of chloroform, 50 μL of methanol and 225 μL of water were added to each sample, and the mixture was separated into two phases aided by centrifugation. The upper phase was removed, and the lower phase was further extracted with 500 μL of methanol/water (10:9, vol/vol). The two portions of upper phase were combined, and 37.5 μL of 1M HEPES was added to neutralize the mixture. The resulting deacylated products were vacuum-dried and reconstituted in water to appropriate volumes for analysis by HPLC using a Dionex BioLC system (Dionex, Westmont, IL). Samples (50 μL) were fractionated on a Partisil 10 SAX analytical column and a Solvecon pre-column using a discontinuous linear gradient solvent system containing 1.7M ammonium formate buffered to pH 3.7 with orthophosphoric acid. The percentage of ammonium formate was increased from 0 to 44% within 40 min, to 60% at 50 min and to 100% at 70 min. The flow rate was set at 1 mL per min. The eluates were collected in test tubes as one-minute fractions, mixed with 5 mL of scintillation fluid (Universol) each, and radioactivity was counted with a Beckman LS5801 scintillation spectrometer (Beckman Instrument, Fullerton, CA).

Analysis of PI fatty acids by gas–liquid chromatography (GLC). In one experiment, the lipid extracts from primary glial cells and DITNC cells were separated by two-dimensional HPTLC (16). Individual lipid fractions were recovered from the plate, and the acyl groups were converted to their fatty acid methyl esters (FAME) by base-catalyzed methanolysis with 0.2N NaOH in methanol. A known

amount of 17:0 methyl ester (Sigma) was added to each sample as internal standard. The FAME were resolved using a Hewlett-Packard gas–liquid chromatograph (HP 5890; Hewlett-Packard, Avondale, PA) with an SP-2330 (30 m × 0.32 mm i.d.) fused silica capillary column (Supelco, Bellefonte, PA). Conditions used for GLC were similar to those described previously (16).

RESULTS

The membrane fractions that were isolated from primary glial cells and immortalized astrocytes (DITNC) were incubated with [^{32}P]ATP. Subsequent analysis of the labeled lipids by HPTLC revealed as many as eight labeled lipid bands (lanes C and D in Fig. 1). For comparison, incubation of SPM and PM from rat brain with [^{32}P]ATP also resulted in a number of labeled lipid bands (lanes E and F) but showed different labeling patterns. In lanes A and B of Figure 1, the migration patterns of lipids from A (primary glial cells) and B [a commercial polyPI standard containing PI, PIP and PIP_2 (Sigma)] are seen after exposing the HPTLC plate to iodine vapors. Most of the nonradioactive lipids migrated to the top half of the plate, whereas the labeled lipids were retained in the lower half (Fig. 1).

The separation of PA, PI and lysoPI by HPTLC was verified with commercial standards. Lane A in Figure 2 shows the migratory pattern of a polyPI standard (Sigma). In lanes B and C, it is shown that the PA standard migrates to a position slightly lower than does PI. In fact, this standard contained an impurity that was later identified as lysoPA. LysoPI (lane E) migrated to a level similar to that of PA. However, when labeled lipid bands 1 and 2 (Fig. 1) were separated using the two-dimensional HPTLC system, radioactivity correlated well with the PA and lysoPA fractions and not with PI or lysoPI (data not shown).

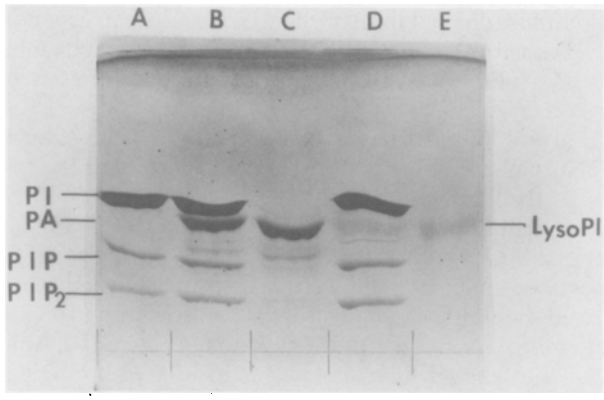

FIG. 2. Separation of polyPI (PI, phosphatidylinositol) standards, phosphatidic acid (PA) and lysoPI by high-performance liquid chromatography. Lipid bands were visualized by charring the plate upon spraying with copper acetate reagent. Bands: (1) PI; (2) PA or lysoPI; (3) PI phosphate (PIP); and (4) PIP_2. Lanes: (A) polyPI standard; (B) polyPI plus PA; (C) PA; (D) polyPI plus lysoPI; and (E) lysoPI.

For further identification of the lipid fractions, especially those that do not correspond to PA, PIP and PIP_2, individual lipid bands were recovered from the HPTLC plate and deacylated. The water-soluble products were analyzed by HPLC. In Figure 3, the radioactive peak at 17–24 min was found to correspond well to the elution profile of glycerol-3-phosphate (G-3-P), the deacylation product of PA. The peak at 25–30 min corresponded to gPIP, the deacylation product of PIP, and the peak at 46–50 min corresponded to $gPIP_2$, the deacylation product of PIP_2. In addition to the elution profile for the [^{32}P]labeled deacy-

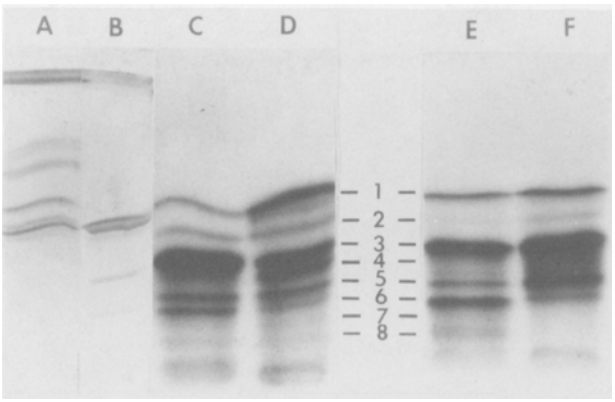

FIG. 1. High-performance thin-layer chromatographic separation of ^{32}P-labeled phospholipids produced by incubation of [γ-^{32}P]adenosine triphosphate (ATP) with membranes isolated from primary glial cells and immortalized astrocytes (DITNC). The data are compared with those for synaptic plasma membranes (SPM) and plasma membranes (PM) from rat brain. Lipids in lanes A and B were visualized by exposure to iodine vapors; lanes C–F were autoradiographs. A, phospholipids in primary astrocyte membranes; B, polyPI standards (Sigma Chemical Co., St. Louis, MO); C, primary glial cells; D, DITNC cells; E, SPM from rat brain; F, PM from rat brain.

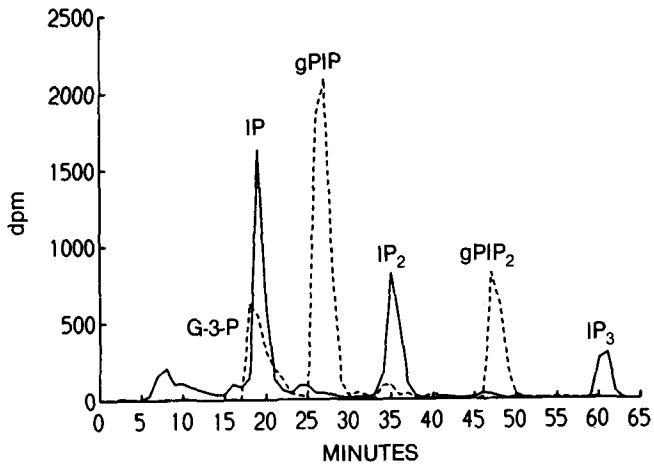

FIG. 3. High-performance liquid chromatography separation of the phospholipids from ^{32}P-labeled SPM upon deacylation (dotted line) and comparison with ^{3}H-labelled inositol monophosphate (IP), inositol biphosphate (IP_2) and inositol trisphosphate (IP_3) standards (solid line). The glycerol-3-phosphate (g-3-P), gPIP and $gPIP_2$ peaks correspond to deacylated PA, PIP and PIP_2 on the high-performance thin-layer chromatography plate, respectively. Abbreviations as in Figures 1 and 2.

lated lipids (dashed line), radioactive peaks corresponding to [^{3}H]inositol-1-phosphate (IP), inositol 1,4-bisphosphate and IP_3 (solid line) are shown in the same figure for comparison (Fig. 3).

Analysis of the deacylated lipids after incubating DITNC cell membranes with [^{32}P]ATP revealed the presence of three major radioactive peaks with elution times corresponding to PA, PIP and PIP_2, respectively (Fig. 4A). When the labeled lipid in band 1 (lane D of Fig. 1) was deacylated and applied to HPLC, the elution profile showed a single radioactive peak corresponding to G-3-P, confirming the identity of this lipid as PA (Fig. 4B). Analysis of the labeled lipid in band 2 also gave a single radioactive peak with an elution time identical to that of G-3-P (Fig. 4B). To further verify that the lipid in band 2 is lysoPA, the lipid was recovered and further resolved by two-dimensional HPTLC. The result obtained indicated that the lipid fraction comigrated with lysoPA (data not shown). The deacylated lipids of bands 3, 4 and 5 each eluted as a single radioactive peak with the elution time of the deacylated PIP peak (Fig. 4C). Based on its migratory pattern in comparison with authentic PI-4-P standard, lipid band 3 was firmly identified as PI-4-P. Lipid band 4 could be due to (i) PI-4-P molecular species different from those in band 3; (ii) PI-3-P; or (iii) lysoPIP. The possibility of lipid band 4 being PI-3-P was eliminated because HPLC eluted a deacylated [^{32}P]PI-3-P sample (provided by Dr. P. Wilden, Pharmacology Department, University of Missouri, Columbia, MO) 2 min earlier than PI-4-P (data not shown). Since lipid band 5 was later confirmed as lysoPIP by incubation of membrane fractions with exogenous lysoPI (see below), lipid band 4 was assigned to PI-4-P by elimination. Lipid band 6 was identified as PIP_2 because its migration rate in HPTLC corresponded well to that of the commercial PIP_2 standard. Analysis by HPLC resulted in the elution of a single radioactive peak with an elution time similar to that of $gPIP_2$ (Fig. 4D). Elution of lipid band 7 also gave a radioactive peak with an elution time identical to that of $gPIP_2$ (Fig. 4D). Consequently, this band is assigned to PIP_2 molecular species.

In order to further clarify the identity of lipid bands 5 and 6, which migrated close to each other in HPTLC, exogenous lysoPI, or PIP, was added to the incubation mixture containing [γ-^{32}P]ATP and a supernatant fraction from DITNC cells. This supernatant fraction probably contained both microsomal membranes as well as cell cytosol. Incubation in the absence of exogenous lipids gave a labeling profile in which PIP was the major lipid band and lysoPIP and PIP_2 were minor lipid bands (lane A, Fig. 5). However, addition of lysoPI to the incubation mixture resulted in an increase in the label in band 5 (lane B), whereas an increase in label in band 6 could be observed after addition of PIP (lane D). When both lysoPI and PIP were added, an increase in label in both lipid bands 5 and 6 could be observed (lane C). In this experiment, it was demonstrated that in addition to labeling of PIP and PIP_2, exogenous lysoPI, or PIP, could decrease labeling of the PA band (Fig. 5). The data in Figure 6 further indicate an increase in labeled PIP_2 with increasing amounts of exogenous PIP added to the incubation system.

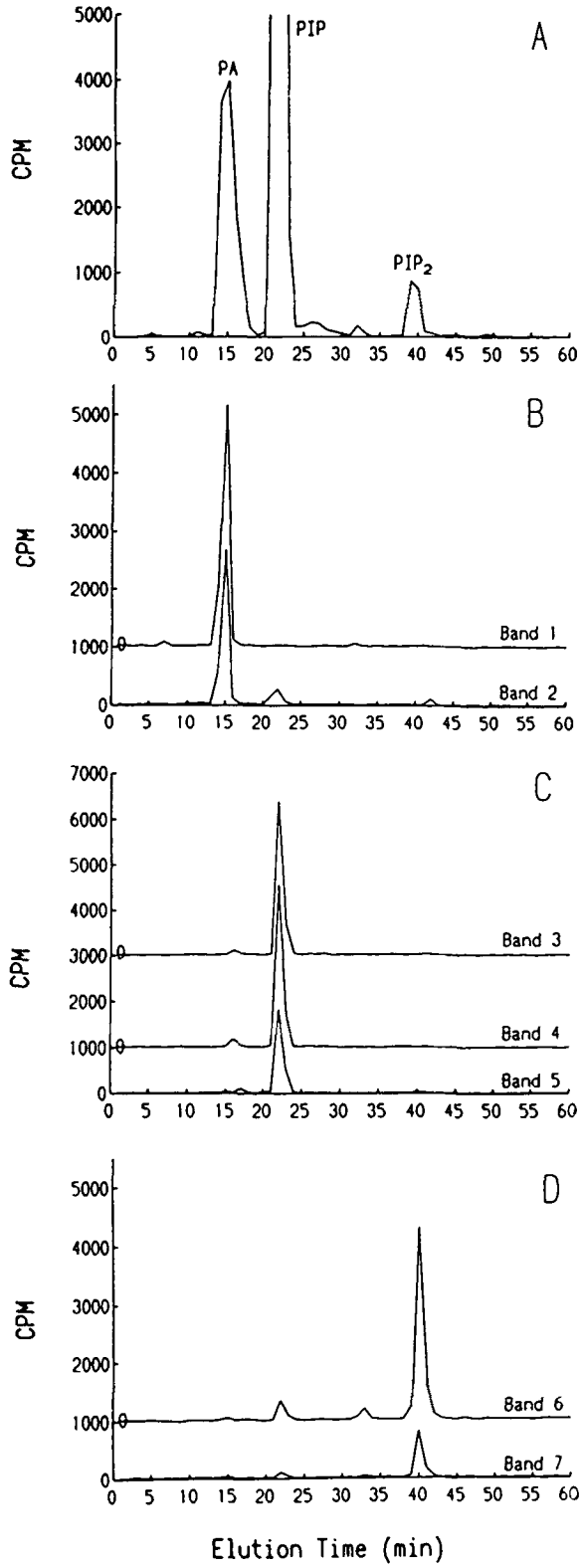

FIG. 4. Identification of ^{32}P-labeled lipid bands after incubation of DITNC cell membranes with [^{32}P]ATP. Panel A: The high-performance liquid chromatography profile of the total phospholipids from DITNC cells; Panel B, lipid bands 1 and 2; Panel C, lipid bands 3, 4 and 5; and Panel D, lipid bands 6 and 7. Lipid band numbers correspond to the numbering used in Figure 1. Abbreviations as in Figures 1 and 2.

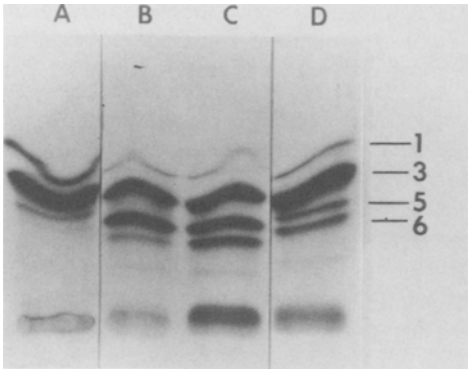

FIG. 5. Incubation of DITNC cell supernatant fraction with [^{32}P]ATP in the presence and absence of exogenous lysoPI and PIP. Band identification: (1) PA; (3) PIP; (5) LysoPIP; and (6) PIP_2. Incubation conditions: Lane A, control, no addition; Lane B, addition of lysoPI (note the increase in labeling of lysoPIP); Lane C, addition of lysoPI and PIP (note the increase in labeling of both lysoPIP and PIP_2); Lane D, addition of PIP (note the increase in labeled PIP_2). Abbreviations as in Figures 1 and 2.

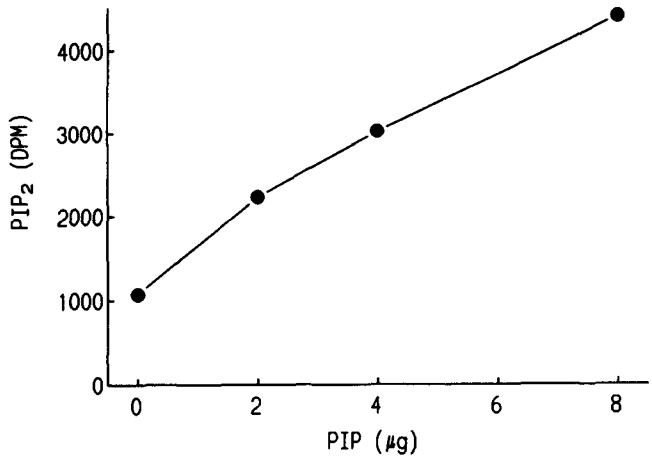

FIG. 6. Increase in labeling of PIP_2 following addition of exogenous PIP to the DITNC cell supernatant fraction. Abbreviations as in Figures 1 and 2.

Although the amounts of PIP and PIP_2 present in the cultured cells were too small to accurately determine their fatty acid composition, it is reasonable to assume that the polyPI have a similar fatty acid composition as does PI, which is present at substantially higher levels. The fatty acid profiles of PI of primary glial cells and DITNC cells are shown in Table 1, together with those of rat brain SPM and PM (12) and rat brain oligodendroglial cells (19). It can be seen that the polyunsaturated fatty acids of PI from the cell cultures are comprised of a considerable amount of 20:3n-9 in addition to 20:4n-6, whereas those from brain membranes contain almost exclusively 20:4n-6 (Table 1).

DISCUSSION

Covalent modification of biological molecules by ATP-dependent phosphorylation is one of the more important mechanisms of cellular regulation. Despite intensive studies on proteins and carbohydrates, the understanding of the enzymes responsible for phosphorylation of lipid mol-

TABLE 1

Fatty Acid Composition of Phosphatidylinositol from Cultured Cells and Rat Brain Membranes[a]

Fatty acids	Glial (%, wt)	DITNC (%, wt)	ROC-1[b] (%, wt)	PM[c] %, wt)	SPM[c] (%, wt)
16:0	6.0 ± 0.7	7.8 ± 1.4	7.8	13.2	11.9
16:1	0.9 ± 0.1	1.9 ± 0.2	3.0	—	—
18:0	37.2 ± 0.8	38.7 ± 6.4	32.8	36.7	35.7
18:1	10.7 ± 1.7	32.5 ± 4.1	28.4	8.8	8.2
18:2n-6	1.4 ± 0.2	4.7 ± 1.8	5.6	—	—
20:3n-9	23.1 ± 1.3	8.4 ± 1.1	12.6	—	—
20:4n-6	17.1 ± 1.2	5.9 ± 1.1	6.0	37.9	39.8
22:3n-3			2.0	—	—
22:4n-6				0.9	1.9
22:6n-3				1.8	2.6

[a]Primary glial cells and DITNC (immortalized astrocytes from rat diencephalon) cells were cultured in 100-mm dishes under the conditions described in the Materials and Methods section. Lipids from cells in each dish were extracted and were separated by two-dimensional high-performance thin-layer chromatography as described (Ref. 16). The phosphatidylinositol fraction was removed, and the fatty acids were converted to their methyl esters for gas–liquid chromatographic analysis. Data are means ± SD of four determinations.

[b]Data for rat oligodendroglial (ROC-1) cells (Ref. 19).

[c]Data for rat brain plasma membranes (PM) and synaptic plasma membranes (SPM) (Ref. 14).

ecules is still limited. Recent studies have demonstrated the importance of sequential phosphorylation of PI to PI-4-P and PI-4,5-P_2 which serves as substrate in the polyPI signal transduction pathway (1–4). On the other hand, a novel class of PI-kinases that phosphorylate PI to form PI-3-P and PI-3,4-P_2 has gained attention, especially as they function in transformed cells (9–11). The latter polyPI isomers seem to play important roles in cellular metabolism and function other than serving as substrates for phospholipase C.

In the present study, incubation of membrane fractions from primary glial cells and immortalized astrocytes (DITNC) with [γ-^{32}P]ATP resulted in the phosphorylation of a number of lipids. Two approaches were used to identify these lipids after separation by HPTLC. First, the chromatographic properties of these labeled lipids were compared with those of authentic standards in one- or two-dimensional HPTLC. Secondly, individual lipid fractions were recovered from the HPTLC plates, deacylated, and the products were analyzed by HPLC. Based on results obtained by the two approaches, it was concluded that lipid bands 1, 2, 3 and 5 (Fig. 1) correspond to PA, lysoPA, PIP and PIP_2, respectively.

In the system we used, incubation of membrane fractions with [^{32}P]ATP also resulted in phosphorylation of DG to PA. This indicates that DG-kinases are present in the membrane preparations, as has been reported for brain (5,6). In most instances, a small amount of lysoPA can be found, probably due to hydrolysis of PA catalyzed by phospholipase A_2. Indeed, incubation of purified PA (labeled with [^{14}C]arachidonic acid) with brain cytosol indicated the susceptibility of this phospholipid to hydrolysis by phospholipase A_2 (unpublished observation).

The possibility that some of the lipid bands of cultured cells appearing in the PIP area could belong to PI-3-P was ruled out after deacylating the lipids and HPLC analysis. The results rather favor the presence of different molecular species of PI-4-P. Indeed, the PI fatty acids from cultured cells contain substantial amounts of 20:3n-9 in addition to 20:4n-6, which is normally present in brain membranes (19,20). The study by Stubbs *et al.* (20) further suggested that fatty acids of phosphoinositides of cultured cells can be readily modified by altering the fatty acids in the culture medium (20). As polyPI are derived by phosphorylation of PI, it is reasonable to assume that the molecular species for all three phosphoinositides are similar under the culture condition (21).

Our results have clearly demonstrated the presence of lysoPIP as one of the phosphorylation products. Careful examination of lipid band 5 indicates that this lipid is present in both the cell and the brain membranes, albeit at different levels (Fig. 1). Because incubation with exogenous lysoPI resulted in an increase in labeled lysoPIP, it is reasonable to conclude that lysoPIP is formed by phosphorylation of lysoPI and not by hydrolysis of labeled PIP. As the lysoPIP band migrated closely to the PIP_2 band in the one-dimensional HPTLC system, it is important that studies with lipid kinases utilize rigorous analytical techniques.

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The Effect of Lidocaine on *de novo* Phospholipid Biosynthesis in the Isolated Hamster Heart

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Lidocaine is used clinically as an antiarrhythmic agent, but its effect on cardiac phospholipid metabolism has not been defined. In this study, hamster hearts were perfused with [1,3-^{3}H]glycerol in the presence of 0.5 mg/mL lidocaine. The incorporation of radioactivities into lysophosphatidic acid, phosphatidic acid, phosphatidylethanolamine, cytidine diphosphate diacylglycerol, phosphatidylinositol, phosphatidylserine, diacylglycerol and triacylglycerol were enhanced by lidocaine treatment, whereas the labelling of phosphatidylcholine was reduced. Analyses of enzyme activities in the heart after perfusion with lidocaine revealed that the activities of phosphatidate phosphatase and acyl-coenzyme A (CoA):1,2-diacylglycerol acyltransferase were enhanced. The presence of lidocaine in the assay did not directly stimulate these enzymes. However, the activity of acyl-CoA:glycerol-3-phosphate acyltransferase was stimulated by lidocaine whereas the activity of cytidine diphosphocholine:1,2-diacylglycerol cholinephosphotransferase was inhibited by lidocaine. We conclude that lidocaine affects the regulation of phospholipid biosynthesis in the heart by both direct and indirect modulation of phospholipid biosynthetic enzymes.
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Phospholipids are the major lipid component of biological membranes (1). In the mammalian heart, the appropriate phospholipid content and composition are important for the maintenance of cellular integrity and proper membrane function, including the conduction of electrical signals across the cardiac membrane (2). Consequently, the biosynthesis and catabolism of phospholipids in the heart must be under rigid control. The participation of certain phospholipids such as phosphatidylinositol as precursors for second messengers in the transduction of biological signals across the cell membrane has been documented (3). More recently, phosphatidylcholine has also been shown to be a precursor of diacylglycerol and phosphatidic acid during agonist stimulation (4). In the last decade, the pathways for the syntheses of the major phospholipids in the heart have been elucidated. However, the mechanisms for the control of these metabolic pathways have not been defined.

A number of amphiphilic compounds have been shown to exhibit regulatory effects on the biosynthesis of phospholipids in mammalian tissues (5–10). Lidocaine is an amphiphilic drug with local anaesthetic properties and is commonly used for the treatment of cardiac arrhythmias. Surprisingly, the effect of lidocaine on the biosynthesis of phospholipids in the heart has not been elucidated. In the present study, the ability of lidocaine to modulate phospholipid biosynthesis in the isolated hamster heart was investigated. Hearts were perfused with labelled glycerol to monitor the *de novo* synthesis of the cardiac phospholipids. Our results show that lidocaine has the ability to modulate the biosyntheses of the major phospholipids in the hamster heart.

MATERIALS AND METHODS

Materials. Palmitoyl-coenzyme A (CoA), DL-α-glycerophosphate, cytidine diphospho(CDP)choline, CDPethanolamine, Tris/HCl, Tris/maleate, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-*bis*(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Lidocaine was obtained from Astra Pharmaceutical Products (Mississauga, Ontario, Canada). [1,3-^{3}H]Glycerol, [1-^{14}C]-oleoyl-CoA, [1-^{14}C]palmitoyl-CoA, [5-^{3}H]cytosine triphosphate (CTP), *myo*-[2-^{3}H]inositol and [^{3}H]acetic anhydride were obtained from Amersham Canada (Oakville, Ontario, Canada). CDP[*methyl*-^{14}C]choline and CDP[1,2-^{14}C]ethanolamine were obtained from NEN division, Dupont Co. (Dorval, Quebec, Canada). 1-Palmitoyl-glycero-3-phosphate, phosphatidic acid (from egg lecithin), phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), phosphatidylserine (bovine brain), phosphatidylinositol (pig liver), dipalmitoyl CDP-diacylglycerol, 1,2-diacylglycerol (pig liver) and triacylglycerol (pig liver) were products of Serdary Laboratories (London, Ontario, Canada). All other chemicals and solvents were of reagent grade and were obtained locally from the Canlab Division of Baxter Co. (Winnipeg, Manitoba, Canada).

Perfusion of the isolated hamster heart. Syrian golden hamsters weighing 120 ± 20 g were used throughout the study. Animals were decapitated and the hearts were removed. The isolated hamster hearts were perfused for 30 to 90 min in the Langendorff mode with Krebs-Henseleit buffer containing 0.3 to 2.0 mM [1,3-^{3}H]glycerol (45 μCi total radioactivity), in the presence or absence of 0.5 mg/mL lidocaine. For some enzyme studies, hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1.0 mM glycerol, with or without 0.5 mg/mL lidocaine.

Determination of uptake and incorporation of [^{3}H]glycerol. Subsequent to perfusion, 10 mL of fresh Krebs-Henseleit buffer was forced through the cannula to remove the remaining perfusate. The heart was cut into pieces, blotted dry, weighed and homogenized in chloroform/methanol (1:1, vol/vol). The homogenate was cen-

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Abbreviations: CDPcholine, cytidine diphosphocholine; CDP-diacylglycerol, cytidine diphosphate diacylglycerol; CDPethanolamine, cytidine diphosphoethanolamine; CoA, coenzyme A; CTP, cytosine triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethylether)*N,N'*-tetraacetic acid; TLC, thin-layer chromatography.

trifuged at 1000 × *g* for 10 min, and the pellet was re-extracted twice with the same solvent. The supernatants were pooled, and a sample of the tissue extract was taken for scintillation counting to determine total glycerol uptake. A 0.25 M KCl solution and chloroform were added to cause phase separation, and samples of the aqueous and organic phases were taken for scintillation counting. The extraction efficiency of labelled phospholipids by this method was 88–90%, and was not affected by lidocaine. The phospholipid and neutral lipid classes in the organic phase were separated by thin-layer chromatography (TLC). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine fractions were separated using chloroform/methanol/15 M aqueous NH_3/water (35:15:2:1, by vol) as developing solvent. The solvent for the separation of phosphatidic acid and lysophosphatidic acid contained chloroform/methanol/HCl (87:13:0.2, by vol) (11). The CDP-diacylglycerol fraction was resolved using a solvent containing chloroform/meth-anol/acetic acid/water (52:20:7:3, by vol) (12). The solvent used for the separation of neutral lipids contained light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (60:40:1, by vol). After visualization by iodine staining, the fractions with migration rates similar to those of authentic standards were scraped into scintillation vials, and their radioactivities were determined by scintillation counting.

Lipid quantification. Phospholipids were quantified by the procedure of Bartlett (13). Diacylglycerol was quantified by acetylation with labelled acetic anhydride by the method of Ishidate and Weinhold (14).

Preparation of subcellular fractions. For the determination of enzyme activities, hearts were removed from the animals and homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl, pH 7.5. Microsomes were prepared from the tissue homogenate by differential centrifugation as previously described (4). Protein concentrations were determined by the method of Lowry *et al.* (15).

Preparation of [^{14}C]phosphatidic acid and determination of phosphatidate phosphatase activity. Phosphatidic acid was obtained from 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerophosphocholine by phospholipase D treatment (16). The substrate (50 μmol, 2000 dpm/nmol) was suspended in 2 mL of diethyl ether, and phospholipase D (1.33 mg) in 2 mL of 100 mM sodium acetate (pH 5.6)/100 mM $CaCl_2$ was added to the substrate suspension in a sealed tube. After 30 min of incubation at 42°C, the reaction mixture was cooled, and 1.33 mg of fresh enzyme was added to the mixture. The reaction mixture was reincubated at 42°C for another 30 min. The reaction was terminated by removing the diethyl ether under N_2. A chloroform/methanol (2:1, vol/vol) mixture (6 mL) was added to cause phase separation. The lower phase was analyzed by TLC with a solvent system of chloroform/methanol/water/acetic acid (50:37:2:3, by vol). The silica gel containing phosphatidic acid was removed, and the phospholipid was extracted from the gel by the method of Arvidson (17).

Phosphatidate phosphatase activity was assayed by determining the production of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycerol from 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphate (18). Phosphatidate phosphatase was found to be more active with a phosphatidic acid containing the 1-palmitoyl-2-oleoyl species than the 1,2-dipalmitoyl species. The assay mixture (100 μL) contained 100 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 0.2 mg of bovine serum albumin, 0.6 mM 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphate (2000 dpm/nmol), 0.4 mM phosphatidylcholine, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.75 mM oleic acid and approximately 0.1 mg protein. Postmitochondrial and cytosolic fractions were preincubated with oleic acid for 20 min at 37°C prior to being added to the reaction mixture. The reaction was initiated by addition of $MgCl_2$, and the mixture was incubated at 37°C for 60 min. The reaction was terminated by addition of 2 mL of chloroform/methanol (2:1, vol/vol). Water was added to cause phase separation, and the labelled diacylglycerol in the organic phase was separated by TLC with a solvent containing light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (60:40:1, by vol). The radioactivity in the diacylglycerol fraction was determined.

Acyl-CoA:glycerol-3-phosphate acyltransferase and acyl-CoA:lysophosphatidic acid acyltransferase assays. Enzyme activities were determined by the procedure of Batenburg *et al.* (19) with the hamster heart homogenate. For the acylation of glycerol-3-phosphate, the assay mixture consisted of 100 mM Tris/HCl (pH 7.5), 1.5 mM *rac*-glycerol-3-phosphate, 90 mM sucrose, 0.5 mg of bovine serum albumin, 1 mM dithiothreitol, 40 μM [1-^{14}C]oleoyl-CoA (4000 dpm/nmol) and 0.1–0.2 mg of protein in a volume of 500 μL. The reaction was initiated by addition of the enzyme, and the mixture was incubated at 30°C for 30 min. The reaction was terminated by addition of 3 mL of chloroform/methanol (2:1, vol/vol). Water was added to cause phase separation. The labelled lysophosphatidic acid in the lower phase was resolved by TLC with a solvent containing chloroform/methanol/15 M NH_3/water (35:15:2:1, by vol), and the radioactivity in the lysophosphatidic acid fraction was determined.

For the acylation of lysophosphatidic acid, the reaction was carried out under the same conditions, except that glycero-3-phosphate was replaced with 0.2 mM 1-palmitoylglycero-3-phosphate (19). The labeled phosphatidic acid formed was separated by TLC, and the radioactivity in the phosphatidic acid fraction was determined.

Acyl-CoA:1,2-diacylglycerol acyltransferase assay. Enzyme activity was assayed by determining the transfer of [1-^{14}C]palmitoyl-CoA to 1,2-diacylglycerol (20) in the hamster heart homogenate. The reaction mixture (0.5 mL) contained 25 mM Tris/HCl (pH 7.4), 50 μM [1-^{14}C]palmitoyl-CoA (2000 dpm/nmol), 2 mM 1,2-diacylglycerol (dispersed by sonication with a Fisher sonic dismembrator fitted with a microprobe; Fisher Scientific, Winnipeg, Manitoba, Canada), 18 mM $MgCl_2$, 0.5 mg of bovine serum albumin and 1 mM dithiothreitol. The reaction was initiated by addition of 0.2–0.3 mg of protein, and the mixture was incubated at 37°C for 10 min. The reaction was terminated by addition of 3 mL of chloroform/methanol (2:1, vol/vol). Water was added to cause phase separation. The product in the organic phase was separated by TLC with a solvent containing light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (60:40:1, by vol), and the radioactivity in the triacylglycerol fraction was determined.

CTP:phosphatidic acid cytidylyltransferase assay. Enzyme activity was determined in hamster heart homogenate and in the microsomal fraction. The reaction mixture contained 50 mM Tris/maleate (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 1.0 mM [5-^{3}H]CTP (10000 dpm/nmol) and 0.5 mM phosphatidic acid (21). The reaction was initiated by addition of the enzyme protein (0.3 mg), and the mixture was incubated at 37°C for 15 min. The reaction was terminated by addition of 2.5 mL of 0.1 M HCl in methanol. After cooling, 5 mL of chloroform was added to the mixture. The chloroform/methanol mixture was washed three times with 10 mL of 2 M $MgCl_2$ (22), and the total radioactivity in the organic phase was determined by scintillation counting. TLC analysis showed that over 98% of the radioactivity in the organic phase migrated to the CDP-diacylglycerol fraction.

Phosphatidylinositol synthase and serine base exchange enzyme assays. Phosphatidylinositol synthase activity was assayed with the hamster heart postmitochondrial fraction by the procedure of Imai and Gershengorn (23). The reaction mixture contained 100 mM Tris/HCl (pH 7.5), 1 mM EGTA, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 0.2% Triton X-100, 0.1 mM CDP-dipalmitoylglycerol and 0.1 mM *myo*-[^{3}H]inositol (5×10^5 dpm/nmol). The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 1 mL of chloroform/methanol/HCl (100:100:1, by vol) and 0.4 mL of 0.9% KCl. After phase separation, the labelled phosphatidylinositol in the organic phase was separated by TLC with a solvent containing chloroform/methanol/15 M NH_3/water (35:15:2:1, by vol), and the radioactivity in the phosphatidylinositol fraction was determined.

Serine base exchange enzyme activity in the microsomal fraction was assayed using labelled serine (24). The reaction mixture contained 10 μM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3), 0.8 μmol phosphatidylethanolamine, 20 nmol [^{3}H]serine (78000 dpm/nmol), 6 μmol $CaCl_2$, 50 μg bovine serum albumin and microsomes in a total volume of 250 μL. The reaction was carried out at 37°C for 15 min, and was stopped with 2 mL of chloroform/methanol (2:1, vol/vol). After phase separation, the radioactivity of phosphatidylserine in the lower phase was determined.

CDPcholine:1,2-diacylglycerol cholinephosphotransferase and CDP ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase assays. Cholinephosphotransferase activity was assayed in the microsomal fraction by determining the transfer of CDP-[^{14}C]choline to diacylglycerol (25). The reaction mixture contained 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.4 mM CDP-[^{14}C]choline (1.0 μCi/μmol), 0.2 mM diacylglycerol (prepared in 0.015% Tween 20) and approximately 0.1 mg protein in a final volume of 200 μL. The reaction was initiated by the addition of labelled CDPcholine, and the mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL of chloroform/methanol (2:1, vol/vol), and water was added to cause phase separation. The organic phase was washed twice with 2 mL of 40% methanol and the radioactivity was determined. Analysis by TLC revealed that over 98% of the radioactivity in the lower phase was in the phosphatidylcholine fraction (25). Ethanolaminephosphotransferase activity was assayed in the microsomal fraction by measuring the transfer of CDP-[^{14}C]ethanolamine to diacylglycerol (26). The reaction mixture contained 10 mM Tris-HCl (pH 8.5), 10 mM $MnCl_2$, 1 mM EDTA, 0.2 mM CDP-[^{14}C]ethanolamine (1.0 μCi/μmol), 0.2 mM diacylglycerol (prepared in 0.015% Tween 20), and approximately 0.1 mg protein in a final volume of 200 μL. The reaction was initiated by the addition of labelled CDPethanolamine, and the mixture was incubated at 37°C for 15 lmin. The reaction was terminated by the addition of 3 mL of chloroform/methanol (2:1, vol/vol), and water was added to cause phase separation. The organic phase was washed twice with 2 mL of 40% methanol, and the radioactivity was determined.

Statistical analysis. Statistical analysis was performed using a two-tailed independent Student's *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

The effect of lidocaine on glycerol uptake and phospholipid biosynthesis. Isolated hamster hearts were perfused with 1.0 mM [^{3}H]glycerol (3 μCi/μmol) for 30 to 90 min in the absence or presence of 0.5 mg/mL lidocaine. Subsequent to perfusion, the total uptake of radioactivity in the heart was determined. The total uptake of radiolabelled glycerol was found to be linear up to 60 min. An enhancement of uptake (88–128%) at all time points (Table 1) was observed when lidocaine was added to the perfusate. In a separate experiment, hearts were perfused with 0.1–2.0 mM glycerol (4.5 μCi/μmol) for 60 min. The uptake of glycerol was found to increase in a concentration-dependent manner between 0.1–1.0 mM (data not shown). The vast majority of the increase in radioactivity uptake was located in the lower (organic) phase of the tissue extract.

TABLE 1

The Effect of Lidocaine on the Uptake of Labelled Glycerol in the Perfused Hamster Heart[a]

	Uptake (10^{-3} × dpm/g wet weight of heart)		
Fraction	30 min (n = 4)	60 min (n = 11)	90 min (n = 3)
Total uptake			
Control	1808 ± 52	3463 ± 575	3826 ± 258
Lidocaine	3977 ± 31[b]	6504 ± 668[b]	8724 ± 1789[b]
Aqueous phase			
Control	948 ± 192	658 ± 113	707 ± 245
Lidocaine	875 ± 23	872 ± 145[b]	973 ± 350
Organic phase			
Control	1057 ± 66	3027 ± 600	3030 ± 799
Lidocaine	3131 ± 124[b]	5926 ± 702[b]	7777 ± 1499[b]

[a]Hamster hearts were perfused for the indicated times with Krebs-Henseleit buffer containing 1 mM [^{3}H]glycerol (3 μCi/μmol) in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the hearts were homogenized in chloroform/methanol (1:1, vol/vol). The tissue extracts were separated into aqueous and organic phases, and aliquots of the two phases were used for radioactivity determination. The results are expressed as mean ± standard deviation for n sets of experiments.

[b]$P < 0.05$.

TABLE 2

The Effect of Lidocaine on the Labelling of Phospholipids and Neutral Lipids in the Hamster Heart[a]

Fraction	Uptake (10^{-3} × dpm/g wet weight of heart)	
	Control	Lidocaine
Lysophosphatidic acid	3.2 ± 0.7 (13)	9.0 ± 2.3[b] (12)
Phosphatidic acid	17.9 ± 3.7 (13)	42.1 ± 8.1[b] (12)
Phosphatidylcholine	172.0 ± 40.5 (9)	103.5 ± 11.9[b] (10)
Phosphatidylethanolamine	174.4 ± 29.4 (14)	448.4 ± 61.7[b] (9)
CDP-diacylglycerol	6.44 ± 1.2 (5)	16.22 ± 4.7[b] (4)
Phosphatidylinositol	35.0 ± 9.9 (13)	61.2 ± 10.2[b] (12)
Phosphatidylserine	22.0 ± 3.1 (13)	41.9 ± 5.5[b] (11)
Diacylglycerol	125.8 ± 29.0 (11)	450.4 ± 110.4[b] (8)
Triacylglycerol	1820.3 ± 508.4 (13)	3995.0 ± 671.1[b] (12)

[a]Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1 mM [^{3}H]glycerol (3 μCi/μmol) in the absence or presence of 0.5 mg/mL lidocaine. After perfusion, the lipid fractions were separated by thin-layer chromatography, and the radioactivity in each fraction was determined. The results are expressed as mean ± standard deviation (number of experiments in parentheses).

[b]$P < 0.05$.

The labelling of phospholipids and neutral lipids in the heart after perfusion was determined. Phospholipid fractions in the lower phase of the tissue extract were separated by TLC, and the radioactivity in each fraction was determined. In a similar manner, neutral lipid fractions in the lower phase were separated by TLC, and the radioactivities associated with diacylglycerol and triacylglycerol were determined. As depicted in Table 2, perfusion of the heart with lidocaine for 60 min caused increases in the incorporation of radioactivities into lysophosphatidic acid (181%), phosphatidic acid (135%), phosphatidylethanolamine (157%), CDP-diacylglycerol (152%), phosphatidylinositol (75%) and phosphatidylserine (90%). The levels of labelling in diacylglycerol and triacylglycerol were also increased by lidocaine perfusion. In contrast, the labelling of the phosphatidylcholine fraction was decreased by 40%.

The determination of lipid contents in the heart after perfusion. Changes in the labelling of lipid fractions after perfusion with lidocaine might be caused by changes in pool sizes of these factions. Analysis of pool sizes of the various lipid fractions showed that lidocaine treatment did not cause any significant changes in the total amounts of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol in the hamster heart (Table 3). In addition, the pool of CDP-diacylglycerol (12.1 ± 0.1 nmol/g wet weight) was unaffected by lidocaine treatment. However, perfusion with lidocaine caused increases in lysophosphatidic acid (50%) and diacylglycerol (67%) levels.

The effect of lidocaine on the activities of enzymes for the biosynthesis of cardiac lipids. The observed changes in the labelling of cardiac lipids might be caused by perturbations in the activities of the phospholipid biosynthetic enzymes.

TABLE 3

The Effect of Lidocaine on the Phospholipid and Neutral Lipid Contents in the Hamster Heart[a]

Fraction	Content (μmol lipid P/g wet weight of heart)	
	Control	Lidocaine
Lysophosphatidic acid	0.06 ± 0.01 (3)	0.09 ± 0.01[b] (3)
Phosphatidic acid	0.34 ± 0.08 (6)	0.28 ± 0.07 (6)
Phosphatidylcholine	11.14 ± 1.39 (6)	11.85 ± 1.12 (6)
Phosphatidylethanolamine	10.41 ± 1.18 (6)	9.61 ± 0.55 (6)
Phosphatidylserine	1.80 ± 0.32 (6)	1.55 ± 0.25 (6)
Phosphatidylinositol	1.23 ± 0.66 (6)	1.07 ± 0.43 (6)
Diacylglycerol[c]	3.29 ± 0.36 (6)	5.49 ± 0.95[b] (5)

[a]Hamster hearts were perfused for 60 min with Krebs-Henseleit buffer containing 1 mM glycerol. After perfusion, the lipid fractions were separated by thin-layer chromatography, and the amount of lipid in each fraction was determined. The results are expressed as mean ± standard deviation (number of experiments in parentheses).

[b]$P < 0.05$.

[c]Diacylglycerol content is expressed as μmol glycerol/g wet weight of heart.

TABLE 4

The Direct Effect of Lidocaine on the Activities of Lipid Biosynthetic Enzymes[a]

Enzyme	Activity (nmol/h per mg protein)	
	Control	Lidocaine (0.5 mg/mL)
Acyl-CoA:glycerol-3-phosphate acyltransferase (n = 3)	2.97 ± 1.13	6.64 ± 1.23[b]
Acyl-CoA:lysophosphatidic acid acyltransferase (n = 5)	1.05 ± 0.31	1.09 ± 0.31
Phosphatidate phosphatase (n = 3)	66.16 ± 19.16	75.16 ± 15.33
Acyl-CoA:1,2-diacylglycerol acyltransferase (n = 4)	36.90 ± 8.10	31.50 ± 10.50
CDPcholine:1,2-diacylglycerol cholinephosphotransferase (n = 3)	69.57 ± 22.19	40.33 ± 14.72[b]
CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (n = 3)	19.72 ± 3.30	15.71 ± 1.35
CTP:phosphatidic acid cytidylyltransferase (n = 3)	0.33 ± 0.07	0.40 ± 0.10
Phosphatidylinositol synthase (n = 3)	0.52 ± 0.10	0.40 ± 0.03
Serine base exchange enzyme (n = 3)	0.13 ± 0.04	0.05 ± 0.03

[a]Enzyme activities were assayed in the presence of 0–0.5 mg/mL lidocaine. The results are expressed as mean ± standard deviation for n sets of experiments, each of which was performed in duplicate. Results were analyzed using a paired *t*-test. CoA, coenzyme A; CDPcholine, cytidine diphosphocholine; CDPethanolamine, cytidine diphosphoethanolamine; CTP, cytosine triphosphate.

[b]$P < 0.05$.

Hence, the direct effects of lidocaine on the activities of these enzymes were determined in subcellular fractions of the hamster heart. As depicted in Table 4, the activity of acyl-CoA:glycerol-3-phosphate acyltransferase was stimulated by lidocaine, which might account for the increased labelling of lysophosphatidic acid. The inhibition of CDPcholine:1,2-diacylglycerol cholinephosphotransferase activity might explain the decrease in the labelling of phosphatidylcholine. The activities of the other phospholipid biosynthetic enzymes were not significantly changed by lidocaine treatment.

The effect of lidocaine perfusion on the activities of the phospholipid biosynthetic enzymes in cardiac subcellular fractions was also examined. Hamster hearts were perfused for 60 min with 1 mM glycerol, in the absence or presence of 0.5 mg/mL lidocaine. Subsequent to perfusion, subcellular fractions of the hamster heart were prepared, and enzyme activities were determined in these fractions. As depicted in Table 5, perfusion with lidocaine did not cause any significant change in the activities of acyl-CoA:glycerol-3-phosphate acyltransferase or CDPcholine:1,2-diacylglycerol cholinephosphotransferase. A small enhancement (24%) of acyl-CoA:1,2-diacylglycerol acyltransferase was detected. There was no change in the total activity of phosphatidate phosphatase in the postmitochondrial fraction; however, the activity of this enzyme in the microsomal fraction was enhanced by lidocaine perfusion.

DISCUSSION

The *de novo* synthesis of phospholipids in mammalian tissues occurs *via* the progressive acylation of glycerol-3-phosphate. Because glycerol-3-phosphate does not readily permeate the plasma membrane, radiolabelled glycerol is usually used as a general precursor for studying phospholipid biosynthesis (6,10,20,27,28). In the hamster heart, the labelled glycerol is taken up rapidly and phosphorylated to glycerol-3-phosphate by glycerol kinase (10). The labelled glycerol-3-phosphate is then acylated to form lysophosphatidic acid and phosphatidic acid followed by the labelling of other phospholipids.

In the present study, perfusion of the isolated hamster heart with 0.5 mg/mL lidocaine elicited an enhancement of

TABLE 5

The Effect of Lidocaine Perfusion on the Activities of Lipid Biosynthetic Enzymes[a]

Enzyme	Activity (nmol/h per mg protein)	
	Control	Lidocaine
Acyl-CoA:glycerol-3-phosphate acyltransferase (n = 3)	2.84 ± 0.95	2.61 ± 0.73
Acyl-CoA:lysophosphatidic acid acyltransferase (n = 3)	1.68 ± 0.38	2.10 ± 0.39
Phosphatidate phosphatase		
Cytosolic (n = 6)	36.67 ± 8.28	35.51 ± 4.10
Microsomal (n = 3)	60.56 ± 4.22	76.20 ± 1.75[b]
CDPcholine:1,2-diacylglycerol cholinephosphotransferase (n = 3)	35.94 ± 12.61	53.84 ±
CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (n = 3)	23.44 ± 2.21	29.08 ± 4.66
Acyl-CoA:1,2-diacylglycerol acyltransferase (n = 3)	41.40 ± 5.34	51.32 ± 3.02[b]
CTP:phosphatidic acid cytidylyltransferase (n = 3)	1.27 ± 0.32	1.16 ± 0.42
Serine base exchange enzyme (n = 3)	1.11 ± 0.36	1.54 ± 0.15

[a]Hearts were perfused with Krebs-Henseleit buffer containing 1.0 mM glycerol in the absence or presence of 0.5 mg/mL lidocaine. Enzyme activities were assayed in the subcellular fractions after perfusion. Results are expressed as mean ± standard deviation for n sets of experiments. Abbreviations as in Table 4.

[b]$P < 0.05$.

glycerol uptake. The drug concentration used in this study was significantly higher than the therapeutic dosage used in the treatment of cardiac arrhythmias (an initial dose of 200 mg administered over 15 min). Increases in the labelling of acidic phospholipids, phosphatidylethanolamine and neutral glycerolipids were detected. The increases in the labelling and the pool size of lysophosphatidic acid indicate that the biosynthetic process of acylation was also elevated. The direct stimulation of the acyl-CoA:glycerol-3-phosphate acyltransferase by lidocaine might provide an explanation for the increase in the labelling and the pool size of lysophosphatidic acid. Although the other phospholipid biosynthetic enzymes were not stimulated by lidocaine, the 2.8-fold increase in the labelling of lysophosphatidic acid would cause an increase in the labelling of phosphatidic acid and, subsequently, the other phospholipids.

The inability of lidocaine to elicit a direct stimulation of the activity of phosphatidate phosphatase did not provide an adequate explanation for the observed increase in the labelling of diacylglycerol. Hence, the modulation of enzyme activity by lidocaine perfusion was also explored. When the heart was perfused with lidocaine, acyl-CoA:glycerol-3-phosphate acyltransferase activity was not affected, but phosphatidate phosphatase and acyl-CoA:1,2-diacylglycerol acyltransferase activities were stimulated. At present, the exact mechanism for the modulation of these enzyme activities by lidocaine in the perfused heart remains undefined. It appears that the increase in the microsomal phosphatidate phosphatase activity did not result from a translocation of enzyme from the cytosol since the activity in the cytosolic fraction was unchanged. Two distinct types of phosphatidate phosphatase are associated with the microsomal fraction (29,30). In this study, we made no attempt in our assays to distinguish between the two enzyme types since both types hydrolyze phosphatidic acid to yield diacylglycerol. The stimulation of the microsomal phosphatase activity (31) may explain the increase in the labelling and pool size of diacylglycerol. The enhancement of acyl-CoA:1,2-diacylglycerol acyltransferase activity might also contribute to the observed increase in the labelling of triacylglycerol.

The decrease in the labelling of phosphatidylcholine was probably caused by a direct inhibition of cholinephosphotransferase activity by lidocaine. It is rather intriguing that similar inhibition of cholinephosphotransferase was not detected in the microsomes of the hamster heart after perfusion with lidocaine. The structural similarity between lidocaine and CDPcholine suggests that inhibition might take place at the substrate level. However, kinetic studies (data not shown) revealed that the inhibition was of the "mixed type," which suggests that lidocaine did not inhibit the reaction in a competitive manner. The fact that the labelling of phosphatidylcholine was reduced during lidocaine perfusion indicates that the direct inhibition of enzyme activity by lidocaine might also be an important mechanism for the regulation of phospholipid biosynthesis in the hamster heart. The differential effect of lidocaine on cholinephosphotransferase and ethanolaminephosphotransferase also suggests that there are separate and independent controls for the biosynthesis of phosphatidylcholine and phosphatidylethanolamine in the hamster heart.

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Effects of Soybean Lipoxygenase-1 on Phosphatidylcholines Containing Furan Fatty Acids

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Naturally occurring tetraalkylsubstituted furan fatty acids (F-acids) were tested as potential substrates for soybean lipoxygenase-1. For this purpose, F-acid methyl ester and phosphatidylcholines containing F-acids at the *sn*-2 position of the glycerol residue were incubated with the enzyme. Oxidation of F-acids only occurs in the presence of linoleic acid as co-substrate. Linoleic acid is converted by lipoxygenase to the corresponding hydroperoxide that oxidizes the F-acid, probably in a radical reaction, to form an unstable dioxoene compound. This intermediate then forms, dependent on pH, unsaturated furanoid acids or isomers with cyclopentenolone structure that can be detected by gas chromatography/mass spectrometry (GC/MS). F-acids located at the *sn*-2 position of a synthetic phosphatidylcholine (PC), containing linoleic acid in the *sn*-1 position, are co-oxidized to a greater extent by incubation with soybean lipoxygenase-1 than are F-acids bound to PC with myristic acid in the *sn*-1 position when subjected to the enzyme in the presence of a great excess of linoleic acid. The results suggest that F-acids may play a strategic role in antioxidative processes in plant cells.

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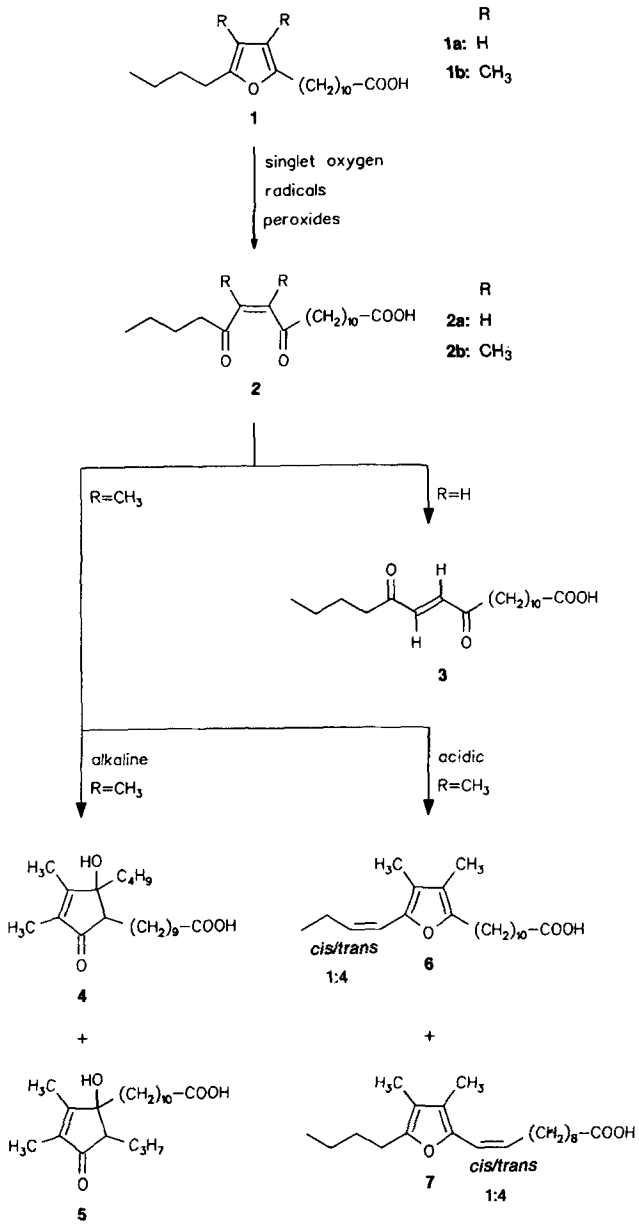
Furan fatty acids (F-acids) (**1**) (Scheme 1), first shown by Glass *et al.* (1,2), to occur in freshwater fish are apparently ubiquitous components of animal (3–6) and plant (7–9) tissues. Biogenesis (10–13) and metabolism (14–17) of F-acids have been studied intensively, but the biological role of F-acids is still obscure. First speculations that F-acids may be involved in animal reproduction rhythm (2) and embryo development (18) could not be confirmed yet. Nevertheless the wide distribution of F-acids and their similarities with physiologically active eicosanoids (19–21) and octadecanoids (22–24) seem to indicate that F-acids are not merely metabolic waste-products.

The furan ring in F-acids is highly reactive (2,25,26), and there is evidence that the oxidative opening of the furan ring yielding 1,4-dioxoenoic acids (**2**) may be of physiological importance. This reaction can be induced by singlet oxygen, organic peroxides and free radicals (27–30) (Scheme 1). Significant differences in the reactivity between naturally occurring tetraalkysubstituted F-acids (**1b**) and α-dialkylsubstituted model compounds (**1a**) were observed: Synthetic F-acids (**1a**), lacking methyl groups in the ß-positions of the furan ring, are oxidized *via* the *cis*-isomer **2a** to the corresponding stable *trans*-dioxoene compound **3** (29). In contrast, it is not possible to isolate analogous products by oxidation of tetraalkylsubstituted F-acids (**1b**): Alkaline extraction of their oxidation products **2b** yields isomers with cyclopentenolone structures **4** and **5** due to intramolecular



SCHEME 1

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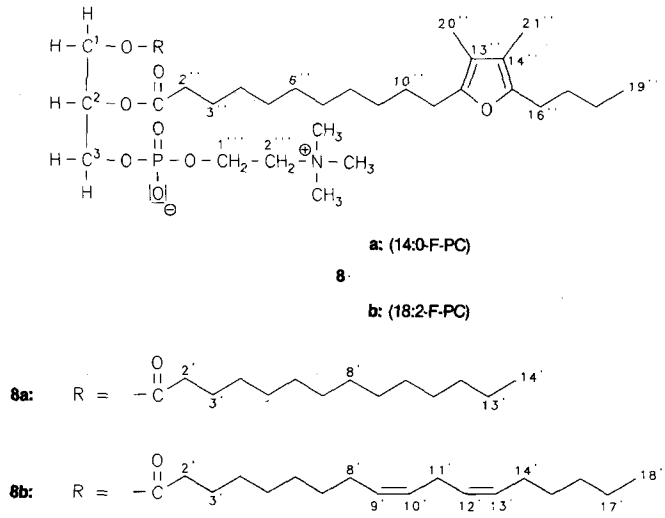
Abbreviations: DMAP, 4-dimethylaminopyridine; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride; F-acid(s), furan fatty acid(s); FID, flame-ionization detector; GC, gas chromatography; LSI–MS, liquid secondary ion mass spectrometry; LOX, soybean lipoxygenase-1; MS, mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NMR, nuclear magnetic resonance; PC, phosphatidylcholine(s); 14:0-F-PC, 1-tetradecanoyl-2-[11-(3,4-dimethyl-5-butyl-furan-2-yl)undecanoyl]-*sn*-glycero-3-phosphocholine; 18:2-F-PC, 1-(*cis*-9,*cis*-12-octadecadienoyl)-2-[11-(3,4-dimethyl-5-butylfuran-2-yl)undecanoyl]-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; Tris-HCl, *tris*(hydroxymethyl)amino-methane hydrochloride.



SCHEME 2

aldol condensation (31) (Scheme 1), while under acidic conditions **2b** reacts to isomeric unsaturated furanoid acids **6** and **7** by recyclization with introduction of a conjugated double bond (32) (Scheme 1).

Originally, the oxidation of F-acids (**1**) to the corresponding dioxoene compounds **2** was assumed to be directly effected by lipoxygenase (29,33). Recently it was demonstrated that dialkylsubstituted F-acids (**1a**) are not substrates of the enzyme (34,35).

In the present study the effects of soybean lipoxygenase-1 (LOX) on naturally occurring tetraalkylsubstituted F-acids (**1b**) were investigated. F-acid methyl ester, as well as phosphatidylcholines (PC) containing tetraalkylsubstituted F-acid in the *sn*-2 position of the glycerol residue, and either myristic acid (14:0-F-PC) (**8a**) or linoleic acid (18:2-F-PC) (**8b**) in the *sn*-1 position (Scheme 2, shown with chemical shift assignments) served as substrate for the experiments.

MATERIALS AND METHODS

Materials. All chemicals were of highest grade available and used without further purification. The solvents, if no other data are given, were of technical standard and freshly distilled before use.

Synthesis of 11-(3,4-dimethyl-5-butyl-furan-2-yl)undecanoic acid methyl ester. The methyl ester of tetra-alkyl-substituted F-acid (**1b**) was prepared following the methods of Rahn *et al.* (36) and Schödel and Spiteller (31) from 3,4-dimethyl-2-butylfuran by Friedel-Crafts acylation with undecanedioic acid monomethylester monochloride (37) followed by Huang-Minlon reduction (38). Purity according to gas chromatography (GC) was 94.2%; R_i = 2360.

*Preparation of 1-tetradecanoyl-2-[11-(3,4-dimethyl-5-butyl-furan-2-yl)undecanoyl]-*sn*-glycero-3-phosphocholine* (**8a**). Tetraalkylsubstituted F-acid methyl ester (15.2 mg, 43 µmol) was saponified with 2 mL 1 M NaOH (methanol/water, 9:1, vol/vol) at 60°C under argon for 90 min (39). After cooling, acidification with HCl (pH 1) and addition of water (50 mL), the acid **1b** was extracted with *n*-hexane/diethyl ether (50 mL; 4:1, vol/vol). The organic layer was washed twice with water (2 × 20 mL) and the organic solvent removed under reduced pressure. The residual yellow oil was evaporated to dryness under reduced pressure with addition of cyclohexane to azeotropically remove water from the sample. Condensation to **8a** was accomplished following a modification of the method by Mena and Djerassi (40): 4-dimethylaminopyridine (DMAP; 8 mg, 65.5 µmol, >98% by GC; Fluka, Neu-Ulm, Germany), 15 mg (32 µmol) 1-tetradecanoyl-*sn*-glycero-3-phosphocholine [99% by thin-layer chromatography (TLC); Sigma, Deisenhofen, Germany] and 9.6 mg (50 µmol) *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; >99%; Fluka) were suspended together with the F-acid (**1b**) in 5 mL dry dichloromethane (p.a.; Fluka). The mixture was stirred for 8 h under argon (Linde, Unterschleissheim, Germany). Excess dichloromethane was removed in a stream of nitrogen (Linde), and the 14:0-F-PC (**8a**) produced was extracted according to Bligh and Dyer (41). The crude product was purified by preparative TLC on Silica Gel 60 PF_{254} (Merck, Darmstadt, Germany) using chloroform/methanol/25% aqueous ammonia (65:35:7, by vol) as solvent system. A single band corresponding to 14:0-F-PC (**8a**) was identified by spraying the dry plates with Zinzadze reagent (42) (R_f = 0.29). Zinzadze reagent detects phosphoric acid by development of blue stain. The silica band was scraped off and eluted with 50 mL chloroform/methanol (1:1, vol/vol). Yield of 14:0-F-PC (**8a**): 16.2 mg (48%). The product was dissolved in benzene to obtain a stock solution that was frozen under argon at –20°C. The concentration of this stock solution was determined to be 7.65 µg 14:0-F-PC/µL. Quantification was done by peak area integration of GC measurements after saponification of a known amount of phospholipid. For that, 9-(3,4-dimethyl-5-butyl-furan-2-yl)nonanoic acid methyl ester was used as internal standard. Liquid secondary ion mass spectrometry (LSI–MS): $[M + H]^+$, *m/z* 786. ^1H nuclear magnetic resonance (NMR) (500 MHz) in C_6D_6/CD_3OD (2:1, vol/vol). δ 0.77 (3H, *t*, *J* = 7 Hz, H-19″), 0.81 (3H, *t*, *J* = 7 Hz, H-14′), 1.08–1.28 (34 H, *m*, H-4′ –H-13′, H-4″ – H-9″, H-18″), 1.44–1.58 (8H, *m*, H-3′, H-3″, H-10″, H-17″), 1.70 (3H, *s*, H-20″/H-21″), 1.72 (3H, *s*, H-20″/H-21″), 2.13 (2H, *t*, *J* = 7.5 Hz, H-2′/H-2″), 2.19 (2H, *t*, *J* = 7.6 Hz, H-2′/H-2″), 2.41 (2H, *t*, *J* = 7.1 Hz, H-11″/H-16″), 2.42 (2H, *t*, *J* = 7.0 Hz, H-11″/H-16″), 2.77 (9H, *s*, N-CH_3), 3.20–3.23 (2H, *m*, H-2‴), 4.06–4.13 (4H, *m*, H-1 and H-1‴), 4.19 (1H, *dd*, J_1 = 12.1 Hz, J_2 = 7.0 Hz, H-3a), 4.47 (1H, *dd*, J_1 = 12.1 Hz, J_2 = 3.1 Hz, H-3b), 5.33–5.38 (1H, *m*, H-2).

*Preparation of 1-(*cis*-9,*cis*-12-octadecadienoyl)-2-[11-(3,4-dimethyl-5-butyl-furan-2-yl)undecanoyl]-*sn*-glycero-3-phosphocholine* (**8b**). 1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine (25 mg, 32 µmol, 99% by TLC; Sigma) was dissolved in 5 mL diethyl ether and 250 µL H_3BO_3 solution (100 mM, pH 7.2) was added. After addition of 0.7 mg phospholipase A_2 (from *Naja mocambique mocambique*, isoenzyme pI = 8.8, lyophilized powder, 1540 units/mg protein; Sigma), which was suspended in 250 µL of buffer solution (100 mM Tris-HCl/4 mM $CaCl_2$,

pH 7.5) (Sigma), the mixture was homogenized with a vortex mixer (Bender & Hobein, Zürich, Switzerland). Then the suspension was shaken for 3.5 h under argon at 26.7°C in a water bath with repeated vortexing. After evaporation of the diethyl ether in a stream of nitrogen, the lysophosphatidylcholine produced was extracted according to Bligh and Dyer (41) (15 mL methanol, 15 mL chloroform, 13 mL 0.88% KCl solution). The organic solvent was removed under reduced pressure and the residue purified by TLC on Silica Gel 60 PF_{254} (Merck) plates using chloroform/methanol/25% aqueous ammonia (65:80:13, by vol) as solvent system. 1-(*cis*-9,*cis*-12-octadecadienoyl)-*sn*-glycero-3-phosphocholine was detected with Zinzadze-reagent (42) and eluted with 30 mL chloroform/methanol (1:1, vol/vol) and 20 mL methanol. Condensation of linoleoyl lysophosphatidylcholine was carried out as described for 14:0-F-PC (**8a**) by dissolving the PC in 5 mL dichloromethane and addition of an excess of *ca.* 19.5 mg F-acid (**1b**) (in 4 mL dichloromethane), 100 mg EDC and 20 mg DMAP. After purification by TLC (R_f = 0.57), the yield of 18:2-F-PC (**8b**) was 5 mg (19%). The 18:2-F-PC (**8b**) was dissolved in benzene, and the concentration of this stock solution was determined to be 13.6 µg 18:2-F-PC/µL as described for 14:0-F-PC. LSI–MS: $[M + H]^+$, *m/z* 838. ^1H NMR (500 MHz) in C_6D_6/CD_3OD (2:1, vol/vol). δ 0.80 (3H, *t*, *J* = 7.4 Hz, H-19″), 0.82 (3H, *t*, *J* = 6.7 Hz, H-18′), 1.10–1.62 (36H, *m*, H-3′ – H-7′, H-15′ – H-17′, H-3″ – H-10″, H-17″ and H-18″), 1.73 (3H, *s*, H-20″/H-21″), 1.75 (3H, *s*, H-20″/H-21″), 1.97–2.02 (4H, *m*, H-8′ and H-14′), 2.15 (2H, *t*, *J* = 7.4 Hz, H-2′/H-2″), 2.22 (2H, *t*, *J* = 7.5 Hz, H-2′/H-2″), 2.44 (2H, *t*, *J* = 7.3 Hz, H-11″/H-16″), 2.46 (2H, *t*, *J* = 7.3 Hz, H-11″/H-16″), 2.73 (9H, *s*, N-CH_3), 2.76–2.80 (2H, *m*, H-11′), 3.17–3.21 (2H, *m*, H-2‴), 4.11–4.17 (4H, *m*, H-1 and H-1‴), 4.24 (1H, *dd*, J_1 = 12.1 Hz, J_2 = 7.0 Hz, H-3a), 4.52 (1H, *dd*, J_1 = 12.0 Hz, J_2 = 3.0 Hz, H-3b), 5.33–5.45 (5H, *m*, H-9′ and H-10′, H-12′, and H-13′, H-2).

Incubation of 11-(3,4-dimethyl-5-butyl-furan-2-yl)undecanoic acid methyl ester. F-acid methyl ester (0.42 mg, 1.2 µmol), dissolved in 0.15 mL of acetone, was added to 5 mL of borate buffer (0.2 M, pH 9). The solution was stirred at 20°C for 1 h in the presence of pure oxygen (Linde) and in the darkness as well as under irradiation with two white fluorescent lamps (Gro-Lux Sylvania 20W; Fa. Feustel, Bayreuth, Germany; and Tungsram F-25 U-White 20W; Tungsram, Vienna, Austria). The experiment was also done under the following conditions: (i) with addition of linoleic acid (0.75 mg, 99% by GC; Fluka); and (ii) with addition of LOX (7 mg, lyophilized powder, 150000 U/mg protein, M_r *ca.* 108000; Fluka); and (iii) with addition of linoleic acid (0.75 mg, 2.7 µmol) and soybean lipoxygenase (7 mg, 64 nmol) to the solution of F-acid methyl ester in borate buffer. After acidification with concentrated HCl (pH 1–2), each sample was extracted with chloroform (2 × 10 mL) and, after dissolving in methanol, esterified with diazomethane in diethyl ether at room temperature. Excess reagent was removed in a stream of nitrogen, and the residual solution was evaporated to dryness under reduced pressure. The extracts were dissolved in benzene (0.5 mL) and analyzed by GC and GC/mass spectrometry (GC/MS). Quantification was done by peak area integration.

Oxidation experiments with 14:0-F-PC (**8a**) *and 18:2-F-PC* (**8b**). The 5.5 µL 14:0-F-PC stock solution (*ca.* 40 µg or 54 nmol 14:0-F-PC **8a**) were evaporated to dryness under a stream of nitrogen. The substrate was suspended in 500 µL borate buffer (0.2 M, pH 9) containing 2 mmol sodium deoxycholate, and this mixture was stirred for 1 h at room temperature under pure oxygen (Linde). The experiment was repeated under addition of 1 mg LOX (Fluka) to the buffer solution. Another incubation was done with 14:0-F-PC (**8a**) by further addition of *ca.* 15 mg (53 µmol) linoleic acid (99%, Fluka) and 1 mg LOX. Incubations with 18:2-F-PC **8b** were carried out with 3 µL 18:2-F-PC stock solution (*ca.* 40 µg/48 nmol 18:2-F-PC **8b**). Reactions were done with and without addition of LOX as described for 14:0-F-PC. After incubation for 1 h, the samples were extracted using a modified Bligh and Dyer procedure (41,43,44) (19 mL methanol, 19 mL chloroform, 17 mL 0.88% KCl solution). The dry extracts were transesterified (45) and, after addition of 9-(3,4-dimethyl-5-butyl-furan-2-yl)-nonanoic acid methyl ester as internal standard, were methylated with diazomethane in methanol/diethyl ether. Excess reagent was removed in a stream of nitrogen and the residue dissolved in benzene (20 µL). Each sample was trimethylsilylated for 12 h in the darkness at room temperature by addition of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA; 20 µL, Macherey & Nagel, Düren, Germany) and analyzed by GC and GC/MS.

GC and GC/MS. GC was done on a Packard model 438S, United Technologies (Delft, The Netherlands), on a 30 × 0.3 mm fused-silica column DB 1 temperature programmed from 100 to 280°C at 2°C/min. Injector and flame-ionization detector (FID) temperatures were kept at 270 and 290°C, respectively. Carrier gas was hydrogen, and the split ratio was 1:30. Quantification was by peak area integration using a Shimadzu (Duisburg, Germany) C-R3A integrator. Retention indices were determined according to Kováts (46) with *n*-alkanes (C_{12} to C_{30}) as reference compounds.

GC/MS measurements were performed on a Finnigan MAT 95 (Bremen, Germany) double-focusing spectrometer with a MAT-ICIS data system. Electron impact mass spectra were recorded at an ionization energy of 70 eV. For GC, an HP (Hewlett-Packard, Böblingen, Germany) 5890 series II instrument was used with a 30 m × 0.3 mm fused-silica column DB 1. The carrier gas was hydrogen, and the temperature was programmed as used for analytical GC.

LSI–MS. LSI–MS mass spectra were obtained on a Finnigan MAT 8500 mass spectrometer using an SS 300 data system. The target surface was bombarded with an energized Cs^+ ion beam (3 KeV) using a Cs^+ gun (AMD Intectra, Beckeln, Germany). The sample was dissolved in 0.3 µL of acetonitrile (Zinsser Analytik, Frankfurt/M., Germany)/0.1% aqueous trifluoroacetic acid (Fluka) (1:9, vol/vol) and the solution added to 0.5 µL glycerol matrix (Sigma). The exchangeable stainless-steel target has a sample area of 2 mm^2.

NMR spectroscopy. ^1H NMR spectra were recorded on a Bruker (Rheinstetten, Germany) AM 500 spectrometer in C_6D_6/CD_3OD (2:1, vol/vol) (Aldrich, Steinheim, Germany). Chemical shifts are reported relative to tetramethylsilane.

RESULTS

Incubation experiments with F-acid methyl ester. The methyl ester of F-acid **1b** was dissolved in borate buffer (pH 9) and the solution stirred under pure oxygen for 1 h either in the darkness or by irradiation with two white fluorescent lamps. The F-acid was recovered unchanged, as it was when linoleic acid was added to the solution. Also no oxidation was observed after incubation of F-acid methyl ester together with LOX, but in absence of linoleic acid. Only if the co-oxidant linoleic acid was added to the mixture, was the F-acid oxidized. The extent of oxidation did not depend on whether the incubation was carried out in the darkness or with illumination. Due to quantification of GC measurements, approximately 30% of the F-acid methyl ester was recovered unchanged, while the rest was converted to the isomeric unsaturated furanoid esters (**6**) and (**7**) during the acidic extraction procedure. Products **6** and **7** were identified by GC/MS, and Table 1 shows the major, analytically important MS peaks. During the incubation, the co-substrate linoleic acid was almost totally oxidized by the enzyme. Apart from several oxidation products (47), *ca.* 1.0% of linoleic acid was recovered after 1 h. These experiments prove that naturally occurring tetraalkylsubstituted F-acids are not substrates of the enzyme lipoxygenase.

Oxidation experiments with 14:0-F-PC (**8a**) *and 18:2-F-PC* (**8b**). The ability of LOX to oxidize doubly unsaturated fatty acids is not limited to free acids and methyl esters (48), but PC are equally susceptible to oxygenation by the enzyme (49,50). This reaction depends on the solubility of the phospholipid, which is mediated by addition of bile salts (49). The rate of oxidation shows a complex dependency on the concentration of bile salts in the aqueous solutions (49). We investigated in a model system the reactivity of PC-bound tetraalkylsubstituted F-acids in the course of phospholipid oxygenation catalyzed by LOX in the presence of sodium deoxycholate.

TABLE 1

Mass Spectral Data of Oxidation Products Derived from Tetraalkylsubstituted Furan Fatty Acid

Compound	MS data *m/z* (%)	
4	277 (100)	182 (69)
	126 (61)	309 (30)
5	167 (100)	366 (19)
	335 (13)	317 (10)
6	163 (100)	348 (97)
	333 (11)	317 (10)
7	191 (100)	348 (97)
	305 (49)	165 (17)

TABLE 2

Oxidation Rate of Furan Fatty Acid (F-acid) Bound to Phosphatidylcholine (PC) During Lipoxygenase-Catalyzed Phospholipid Oxygenation[a]

Incubation	Recovered F-acid (%) Control	Recovered F-acid (%) With LOX[b]	Oxidized F-acid (% conversion) (Control = 100%)
14:0-F-PC	88.6 ± 4.6	88.5 ± 1.7	—
14:0-F-PC + 18:2	86.5 ± 5.0	48.6 ± 3.9	43.8
18:2-F-PC	92.7 ± 0.9	42.7 ± 6.6	53.9

[a]Data were obtained by peak area integration of gas chromatography measurements using an internal standard for quantification. Values are shown as mean ± SD (n = 3).

[b]LOX (soybean lipoxygenase), lyophilized powder, M_r ~ 108.000, 150.000 U/mg protein.

In control experiments (Table 2) designed to test the oxidative stability of the substrate, solutions of 14:0-F-PC (**8a**), 18:2-F-PC (**8b**) or 14:0-F-PC and added linoleic acid were stirred for 1 h under an oxygen atmosphere in the absence of lipoxygenase. After alkaline transesterification, F-acids were recovered in high yields ranging from 86.5 to 92.7% as judged by integration of GC peak areas (Table 2). Due to oxidation during the extraction procedure, the residual amount of F-acids was oxidized to the two corresponding cyclopentenolone isomers **4** and **5** as confirmed by GC/MS (Table 1).

When 14:0-F-PC (**8a**) was subjected to LOX-induced oxygenation, no reaction occurred, and the same amount of F-acid (*ca.* 88.5%) was recovered as in the control experiment without added enzyme (Table 2).

By contrast, after addition of LOX to the solution of 18:2-F-PC, more than 50% of the F-acid was oxidized (Table 1). Correspondingly higher amounts of the cyclopentenolone isomers **4** and **5** were detected after the alkaline extraction procedure together with several oxidation products of linoleic acid (47). About 3.8% of linoleic acid originally bound to PC was recovered in this experiment.

After incubation of a mixture of 14:0-F-PC (**8a**) and a thousandfold excess of linoleic acid with LOX, a lower yield of F-acid oxidation products (*ca.* 44%) was seen than was observed by incubation of 18:2-F-PC (**8b**) alone with LOX (Table 2). But the oxidation rate of linoleic acid was much higher than that measured when 18:2-F-PC was incubated with LOX. Only *ca.* 2% of the added linoleic acid were recovered.

DISCUSSION

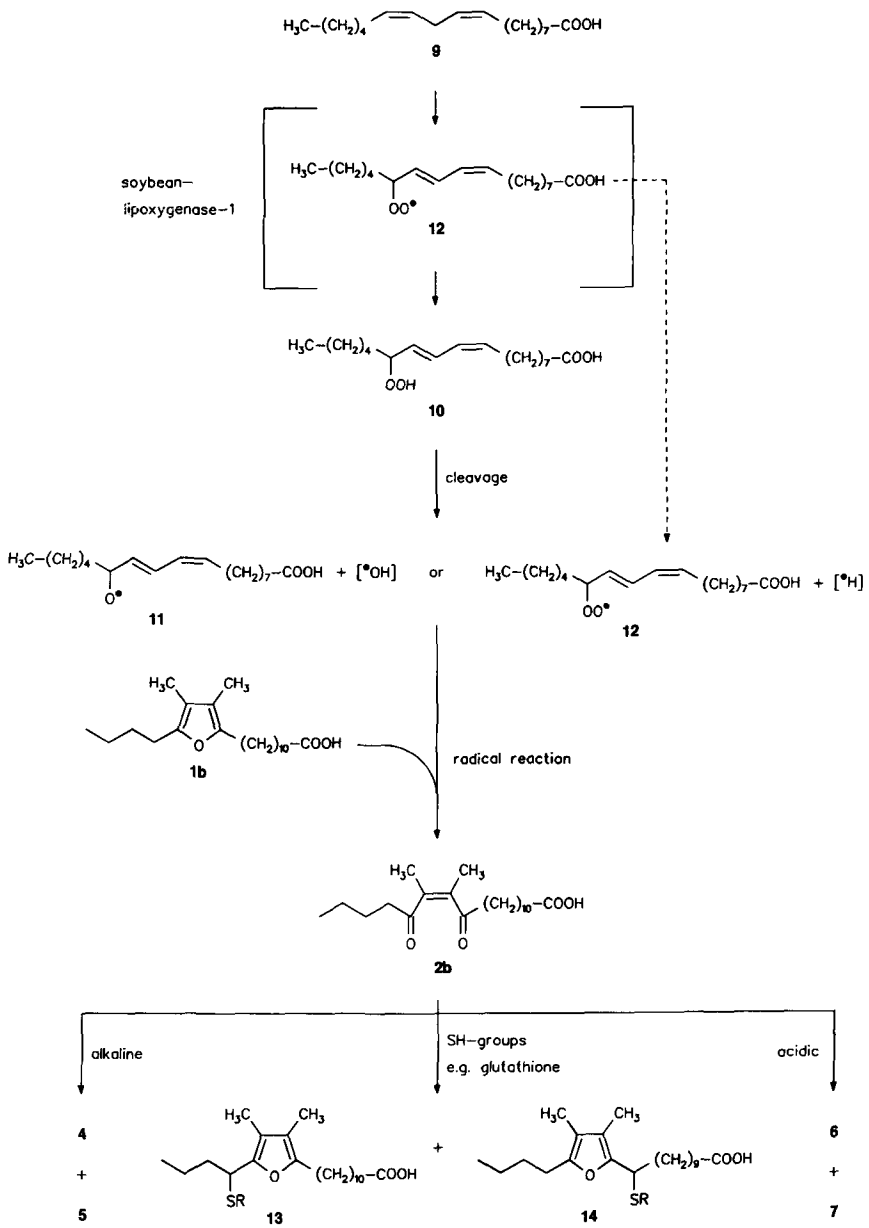
Recently, Zabolotsky *et al.* (34) have shown that dialkyl-substituted F-acids (**1a**) are not attacked by lipoxygenase. Studies in our laboratory have revealed that F-acids lacking methyl groups in ß-position of the furan ring (**1a**) are oxidized in the course of the reaction of LOX with linoleic acid (**9**) (35). Oxidation also occurs by addition of preformed linoleic acid hydroperoxide to the F-acid (35). The reaction can be suppressed by inclusion

of EDTA into the reaction system. This substance is well known for complexing of transition metal ions, especially Fe^{2+}, which induce the decomposition of the hydroperoxide. This behavior is a strong indication for a radical induced transformation of F-acids (35). Only small amounts of oxidation products (**3**) (<1%) could be detected due to the relatively low reactivity of the furan ring in dialkylsubstituted F-acids (35).

In the present paper we demonstrate that tetraalkylsubstituted F-acids (**1b**), either as their methyl ester or when incorporated in PC (**8**), are oxidized by LOX only in the presence of substrates of the enzyme, e.g., linoleic acid. Under these reaction conditions, tetraalkylsubstituted F-acids (**1b**) were converted to the corresponding intermediate dioxoene compounds (**2b**) in yields of *ca.* 50% when bound to PC and *ca.* 70% when present as methyl ester. Thus tetraalkylsubstituted F-acids do not serve as enzyme substrates, but they are co-oxidized much more readily in the course of the lipoxygenase reaction than are the dialkylsubstituted F-acids.

These results are in agreement with the previous observation that F-acids with four substituents on the furan ring are capable of scavenging free radicals as they are generated during oxidation reactions, while dialkylsubstituted F-acids showed only little reactivity in this respect (51). This susceptibility of the furan ring to oxidation led to the assumption that F-acids may serve as antioxidants in biological systems (51,52).

Based on this, we propose the reaction sequence for the co-oxidation of F-acids as illustrated in Scheme 3 to describe the biological relevance of F-acids. The biosynthetic sources of F-acids are plants (9,53), where they



SCHEME 3

are predominantly bound to phospholipids (54). For instance, in the course of cell injury, plant cells liberate *via* phospholipases polyunsaturated fatty acids, e.g., linoleic acid (**9**) from phospholipids (55). These can be converted by activated lipoxygenases (55) to the corresponding hydroperoxides (**10**) (56). The primary oxidation products (**10**) may be cleaved either to the alkoxyl- (**11**) or peroxyl-radicals (**12**) (47,57). While the alkoxyl radial cyclizes rapidly to an epoxyallylic radical (58) the lifetime of the peroxyl radical is quite long (59), and therefore it is able to oxidize furans (60). We speculate that one of these species oxidizes tetraalkylsubstituted F-acids (**1b**) to the dioxoene compounds (**2b**). The latter are unstable and are further converted to other products, dependent on the pH of the medium (33) (Scheme 3).

Alternatively, during enzymatic hydroperoxide formation, highly reactive peroxyl radicals (**12**) are formed (61). In a type-1 lipoxygenase reaction, the substrates seem to have relatively good mobility when bound to LOX (62). Based upon electron spin resonance measurements, the motion of intermediate peroxyl radicals was found not to be restricted during enzymatic fatty acid oxidation (61). It appears that the lifetime of the intermediate peroxyl radical is possibly long enough to react with F-acids (**1b**) producing the corresponding dioxoenoic acids (**2b**), which are further converted as described above (Scheme 3).

To simulate the reactivity of F-acids bound to cell membranes, we investigated the co-oxidation of F-acids bound to phospholipids during enzymatic oxidation with lipoxygenase. The synthetic model compounds 14:0-F-PC and 18:2-F-PC were incubated with the enzyme. Due to the increased oxidation rate of 18:2-F-PC as compared to the oxidation of 14:0-F-PC and a great excess of linoleic acid with LOX (Table 1), our experiments strengthen the hypothesis of a possibly favored intramolecular mechanism. The radical intermediates **11** or **12** may attack the adjacent F-acid in PC more easily than F-acid of another PC molecule, producing a dioxoene-compound (**2b**), which could further react to products **4–7**.

The reactions of the chemically highly reactive intermediate dioxoene compounds (**2b**) (63,64) with thiols, e.g. cysteine or glutathione may be of physiological importance. In the course of these reactions, isomeric thio ethers (**13**) and (**14**) are formed (33,65) (Scheme 3). Therefore, the oxidative opening of the furan ring may introduce changes in structure of cell proteins by reaction with amino acids containing a sulfhydryl group. Thus, the reaction sequence discussed could play a crucial part in the plant defense system against cell injury.

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Cholesterol Secretion from Hepatocytes Induced by Triacylglycerol and Apolipoprotein E

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The mechanism for the increase in plasma cholesterol levels in cholesterol-fed rats following chylomicron transport was investigated in intact animals, in isolated perfused liver, and in hepatocytes in monolayer cultures. Intravenous administration of egg phosphatidylcholine in amounts greater than those required to cause a plasma cholesterol response when given as chylomicrons was without effect. This makes it unlikely that increased plasma cholesterol levels resulted from the recruitment of tissue cholesterol by the plasma chylomicron phospholipids that persisted in the plasma after triacylglycerol clearance. The hepatic origin of the increased plasma cholesterol levels was directly confirmed by two hepatic perfusion experiments. When cholesterol-fed rats received intravenous chylomicrons prior to isolated hepatic perfusion, more cholesterol was secreted by the liver than when the rats were injected intravenously with buffer. Perfusion of apolipoprotein E (apo E)-rich triacylglycerol emulsions through the livers also enhanced cholesterol secretion. The increase in hepatocyte cholesterol secretion seen with cholesterol-fed rats was also noted in monolayer cultures following incubation with apo E rich-triacylglycerol emulsions. The apolipoprotein or the emulsion alone, or apo E-rich phosphatidylcholine liposomes, had no effect. The data confirm previous indirect observations that the liver is the source of cholesterol that appears in plasma following transport of chylomicrons or following a lipid-rich meal in cholesterol-fed rats. The data also re-emphasize the importance of providing apo E with triacylglycerol emulsions to initiate secretion of lower density lipoproteins by the liver.

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Chow-fed rats transport plasma cholesterol primarily in high density or alpha lipoproteins. Cholesterol feeding provokes a modest increase in plasma cholesterol concentration as well as a lipoprotein shift to lower density (d < 1.063) lipoproteins with broad beta mobility (1). This lipoprotein response to cholesterol feeding is only partially understood (2,3). Livers from cholesterol-fed rats have considerably more cholesterol, primarily as cholesteryl esters, than do livers from chow-fed animals, and they secrete more lower density/broad beta lipoprotein cholesterol during isolated hepatic perfusion. The increased cholesterol intake would also reduce the number of low density lipoprotein (LDL) receptors and thus limit LDL catabolism (3).

Human plasma cholesterol concentrations and lipoprotein patterns appear to be influenced more by the amounts and types of triglycerides in the diet than by dietary cholesterol (4). This critical effect of plasma chylomicron flux on plasma LDL cholesterol concentration in humans is not yet well understood (5). Aside from providing a vehicle for the delivery of intestinal cholesterol to the liver for storage, it is likely that the influx of chylomicron remnants signals to the liver to alter plasma lipoprotein flux.

We have observed (6,7) a potent effect of chylomicrons or of particulate triacylglycerol emulsions on plasma cholesterol concentrations in cholesterol-fed rats. Six hours after the plasma clearance of the particulate triacylglycerols, a large (often 2–3-fold) increase in lower density (d < 1.063) lipoprotein cholesterol levels was observed. This was only noted in rats that respond to dietary cholesterol with appreciable increases (greater than threefold) in hepatic cholesterol levels. The effect was unrelated to the inevitable hepatic influx of free fatty acids following triacylglycerol clearance, and did not occur in the anhepatic cholesterol-fed rat (6). These indirect data indicate that the liver may be responsible for the cholesterol increase following plasma particulate triacylglycerol clearance. To test this, a more direct investigation into the role of liver in these processes was undertaken using the isolated perfused liver and hepatocyte in monolayer cultures. The results described here are consistent with our previous suggestion that hepatic secretion is responsible for plasma cholesterol increases and suggest that this secretion is triggered by the final step in plasma triacylglycerol clearance, the uptake of remnants by the cholesterol-loaded liver.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures. Male Sprague-Dawley rats (250–400 g) were purchased from Charles River (Raleigh, NC). The rats were maintained either on conventional chow or on chow supplemented with 2% cholesterol (ICN Chemicals, Costa Mesa, CA) under a 6 a.m.–6 p.m. light cycle. Chylomicrons, triolein (Sigma, St. Louis, MO) emulsions, or egg phosphatidylcholine (Avanti, Alabaster, AL) dispersions were injected through the penile vein with the rats under light pentobarbital anesthesia. The rats were bled from the tail vein. Chylomicrons were obtained from the mesenteric lymphatics after duodenal infusion of 50 mg of monoolein (Sigma) and 50 mg of oleic acid (Sigma) in 5 mL of phosphate-buffered (pH 7) isotonic saline (PBS) using standard procedures (8).

Recirculating *in vitro* liver perfusion was performed by the technique of Mortimore (9). Cholesterol-rich livers were perfused at 2.5 mL/min/g with 3.5% bovine serum albumin and 10% canine erythrocytes in 200 mL Krebs bicarbonate buffer (pH 7.4) at 37°C. The erythrocytes were washed extensively with saline prior to use.

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Abbreviations: Apo E, apolipoprotein E; FBS, fetal bovine serum; HDL, high density lipoproteins; LDL, low density lipoprotein; MEM, minimal essential medium; PBS, phosphate buffered saline; P/C, phosphatidylcholine/cholesterol; P/C-E, P/C-apoE; VLDL, very low density lipoprotein.

Bile flow was measured to monitor the functional state of the liver, and values ranged from 0.4–0.6 mL/h for the 6-h perfusion. Two hours prior to the perfusion, some of the rats were injected with 20 mg of triolein in the form of an egg lecithin/triolein emulsion in 2 mL PBS, while controls received 2 mL PBS. In other liver perfusions, 20 mg of the triolein emulsion and 2 mg of rat apolipoprotein E (apo E) were added to the perfusate 2 h after the perfusion was begun. Aliquots of the perfusate were obtained at half-hour intervals to assay cholesterol mass and lipoprotein distribution.

Cell culture. Hepatocytes and Kupffer cells were obtained from cholesterol-fed rats by a modified (10) collagenase perfusion of the liver as previously described (11). After perfusion of 250 mL of calcium-free [2-hydroxyethyl]piperazine [2-ethanesulfonic acid] (pH 7.4) buffer and 250 mL of 0.05% collagenase, cells were freed and filtered at 4°C. The cells were spun in a Sorvall RC2-B centrifuge (Newton, CT) using an SS-34 rotor at 500 rpm for 2 min at 4°C and then washed three times with calcium-free buffer. An aliquot of the cells was applied to 45% Percoll and centrifuged at 16,000 rpm for 20 min in the Sorvall centrifuge to generate a d 1.1016–d 1.139 gradient. Banding of cells occurred according to cell cholesterol contents ranging from 160 to 20 µg/mg cell protein. The cells were centrifuged out of Percoll into Eagle's minimal essential medium (MEM) with 5% fetal bovine serum (FBS), counted in a hemocytometer, and assayed for viability (11).

Approximately 1.2×10^6 hepatocytes were plated per 35-mm collagen-coated tissue culture dish. The culture medium was 1 mL of MEM containing nonessential amino acids, 10% FBS, 10^{-7} M insulin and 2 µg gentamycin sulfate. Cultures were changed after 2 h to MEM without FBS and maintained in a 95:5% O_2/CO_2 atmosphere at 37°C for up to two days prior to study. Experiments were initiated only after it was demonstrated that apo E enhanced the entry of chylomicrons into the cells (11).

Experiments were initiated by adding chylomicrons, or triolein emulsions, to the MEM at a concentration of 200 µg/mL with or without rat apo E (100 µg/mL), or by adding rat apo E alone (100 µg/mL). The medium was changed after 2 h and assayed for free and total cholesterol. After these initial 2-h incubations, the medium was changed to MEM at 2 h, and again at 12 h, and assayed for free and total cholesterol mass and for radioactivity. Cells were also harvested and assayed for free and total cholesterol, as well as protein.

Apolipoprotein preparation. Apo E was prepared from rat plasma d < 1.21 lipoprotein (12) that had been delipidated with 150 mL of ethanol/diethyl ether (3:1, vol/vol) after dialysis against 0.1% ethylenediaminetetraacetic acid and lyophilization. The nitrogen-dried residue was dissolved in 0.05 M NaCl, 2 mM phosphate pH 7.4 and 5 M urea, and then applied to a heparin affinity column to separate apo E from the other apo-lipoproteins, as previously described (13). Rat apo-lipoproteins A-I and C-III were prepared from the heparin-unbound fraction by sequential molecular sieving (4 M guanidine Sephacryl 300) and diethylaminoethyl cellulose chromatography (14). The purity of the isolated apoproteins was verified by sodium dodecylsulfate–polyacrylamide gel electrophoresis (15). Triolein emulsions, egg lecithin and egg lecithin/free cholesterol (1:1 molar ratio) liposomes were prepared by sonication as previously described (16).

Chemical assays. Total cholesterol mass was determined in media and hepatocytes by both a colorimetric method (17) and by a modified enzymatic assay (Wako Chemicals, Dallas, TX). Free cholesterol was also determined by a modified enzymatic assay (Wako Chemicals). The media and cells were extracted by the Dole method (18), and the organic phases dried under N_2. The residues were then reconstituted with 10 µL of isopropanol and 1 mL of 0.15 M Tris (pH 7.0) 0.1% *p*-chlorophenol containing 0.13 U of both cholesterol ester hydrolase and cholesterol oxidase with 2.4 U of peroxidase, and 4-aminoantipyrine. A standard curve for cholesteryl ester ranging from 2.5–20 µg cholesterol was run in each series along with a laboratory standard. The mixture was incubated for 5 min at 37° and then read at room temperature in a Gilford 230 spectrophotometer at 505 nm. Free cholesterol mass was assayed similarly without use of cholesterol ester hydrolase.

Plasma, perfusate and media lipoproteins were separated into very low density (VLDL; d < 1.006), (LDL; d 1.006–d 1.063), and high density (HDL; d 1.063–d 1.21) by a previously described (12) ultracentrifugation method. After dialysis with PBS, the total d < 1.21 lipoproteins were separated into supernatant and lower fractions by heparin/Mn^{++} precipitation (19). The total d < 1.21 lipoproteins were also separated into heparin/Sepharose-bound and -unbound fractions, as previously described (13). All data are expressed as means ± standard errors; significances were determined by two-tailed Student's *t*-test.

RESULTS

After intravenous injection of 15 mg of chylomicron triacylglycerols into fasted (10 h) male rats, which had been fed cholesterol for six weeks, plasma cholesterol levels increased to more than twice the baseline level (Fig. 1, top panel). Chylomicron clearance was followed by a 5–6 h latency period with stable plasma cholesterol levels prior to the subsequent increase. At least 7.5 mg of cholesterol must have been transferred to plasma (assuming a plasma volume corresponding to 4.5% body weight) to produce the observed increase. Prior to injection, 76 ± 7% of total plasma cholesterol was in ester form, and at the peak plasma level 69 ± 11% (not significantly different). A 15-mg intravenous bolus of an egg phosphatidylcholine dispersion, equivalent to about five times the injected chylomicron phospholipid amount, did not produce the chylomicron effect (Fig. 1, bottom panel). Only a significant gradual increase in free cholesterol occurred, with no change in total cholesterol.

Cholesterol-fed rats (8 wk) were given 20 mg of chylomicron triacylglycerols or an equal volume of PBS through the penile vein 2 h prior to the subsequent *in vitro* liver perfusion. For the first 2 h of perfusion, the

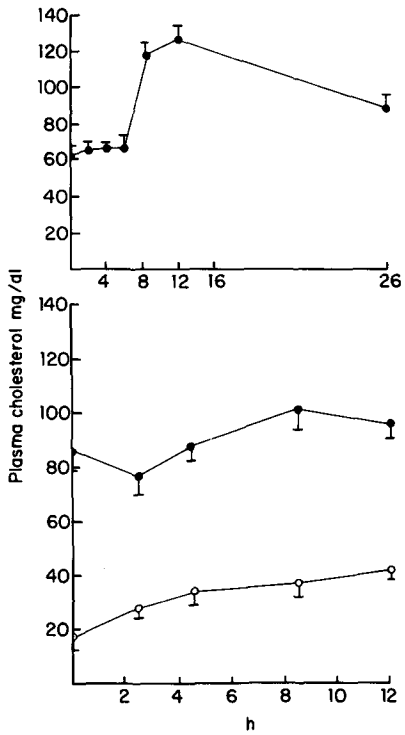

FIG. 1. Top panel: plasma total cholesterol concentration (±SE) in five rats fed a 2% cholesterol supplemented diet for six weeks following intravenous chylomicron administration (15 mg triglyceride) at 0 h. All concentrations after 6 h are significantly ($P < 0.02$) greater than the preinjection (0 h) values. Bottom panel: plasma total (●) and free (○) cholesterol concentrations (±SE) in four of the cholesterol-fed rats used for the chylomicron studies (top) following 15 mg of intravenous egg phosphatidylcholine. All free cholesterol concentrations after the phospholipid injection were greater ($P < 0.05$) than the preinjection (0 h) values.

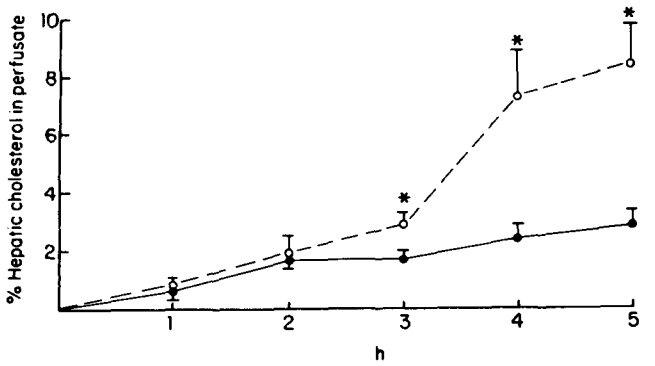

FIG. 2. The fraction of hepatic cholesterol appearing in the perfusate of an isolated *in vitro* perfused liver 2 h after intravenous chylomicron administration (20 mg triglyceride) (○) (n = 5); or buffer (●) (n = 6). The mean (SE) hepatic cholesterol was 34 (12) mg/g liver for the chylomicron injection and 29 (10) mg/g liver for the buffer injection. A significantly ($P < 0.02$) greater fraction of hepatic cholesterol was recovered in the perfusate of the chylomicron-injected than the buffer-injected group at 3, 4, and 5 h (*).

increase in perfusate cholesterol was similar (Fig. 2) for chylomicron and PBS injected rats. In the final 3 h, the perfusates of chylomicron-injected rats were significantly richer in cholesterol than were the controls. After 5 h, cholesteryl esters amounted to 68 ± 8% of the perfusate total cholesterol in the chylomicron-injected rats (n = 5) which was similar to the value (71 ± 4%) found in controls (n = 5). In both, after 5 h, more than 78% of the perfusate cholesterol was associated with heparin/Mn^{++}-precipitable lipoproteins. These 5-h perfusate lipoproteins were ultracentrifuged at d 1.006, d 1.063 and d 1.21. The mean (±SE) percentage distributions of perfusate lipoprotein cholesterol in buffer-injected rats (n = 3) was 16 ± 7, 39 ± 11 and 44 ± 12%, which was similar to the values measured for the perfusates of chylomicron-injected rats (n = 3), i.e., 14 ± 6, 47 ± 13 and 39 ± 14% in HDL, LDL and VLDL, respectively. The LDL and VLDL of both groups of rats had broad beta electrophoretic mobility.

Monolayer cultures of cholesterol-rich hepatocytes from rats fed a cholesterol-enriched diet responded to a soy triacylglycerol emulsion supplemented with rat apo E (Table 1). A significant increase in media cholesterol levels was observed during the 2-h incubation with the apo E-supplemented triacylglycerol emulsion when compared to cells maintained in unsupplemented media, or cells in media that contained only the emulsion, apo E or Na oleate. After the media were removed, the cells were washed with PBS and incubated with MEM alone for 12 h. The cells exposed to the apo E/triacylglycerol emulsions had significantly more media cholesterol than con-

TABLE 1

Induction of Cholesterol Secretion by Hepatocytes in Monolayer Culture[a]

Additions	μg Medium cholesterol/mg cell protein	
	2 h	14 h
None	2.5 (0.4)	5.7 (0.8)
Emulsion 200 μg triacylglycerol (TG)/mL	2.9 (0.3)	6.4 (1.1)
Emulsion 200 μg TG/mL plus apo E 100 μg/mL	5.7 (0.9)[b]	16.3 (2.0)[c]
Apo E 100 μg/mL	2.1 (0.2)	4.0 (0.3)
Na oleate 0.75 mM	3.6 (0.4)	4.5 (0.6)

[a]The results are the means (SE) of quadruplicate determinations of medium total cholesterol at 2 and 14 h after incubating monolayer cultures of cholesterol-rich hepatocytes [88 ± 18 μg/mg (n = 8) cell protein] from rats fed a 2% cholesterol diet for seven weeks, with the triacylglycerol-rich fraction of a commercial soy oil emulsion and/or rat apolipoprotein E (apo E) in minimal essential medium (MEM). The added emulsion contained less cholesterol than was detectable by the assay. The monolayers were maintained on fetal bovine serum for 36 h before apo E response was seen (Ref. 11).

[b]The media of the hepatocytes exposed to emulsion and apo E contained significantly more cholesterol at 2 h than the unsupplemented media (at $P < 0.02$), or the solely emulsion ($P < 0.05$) or apo E ($P < 0.01$) exposed hepatocytes.

[c]The 14-h media (which had been changed to MEM after the initial 2 h incubation) of hepatocytes exposed to emulsion and apo E contained significantly more cholesterol than controls or emulsion exposed cells ($P < 0.01$) or cells solely exposed to apo E ($P < 0.001$). The percentages of free cholesterol (29 ± 7) in this media was not different from those in the 14-h control media (35 ± 9) without additions.

TABLE 2

Changes in Perfusate Cholesterol Levels upon Infusion of an Apo E-Rich Triolein Emulsion[a]

Perfusion	μg Cholesterol/mL/h	
	Before	After
1	1.88 (0.09)	2.68 (0.13)[b]
2	0.92 (0.22)	2.00 (0.22)[c]
3	1.23 (0.41)	2.43 (0.74)
4	0.46 (0.14)	1.00 (0.10)[d]
Control	0.88 (0.24)	0.94 (0.34)

[a]Cholesterol-rich liver (28–76 mg/g liver) was perfused with 100 mL of Krebs-Ringer bicarbonate buffer containing 30 mg bovine serum albumin/mL and a 10% hematocrit of washed canine erythrocytes. For the initial 2-h perfusate, total cholesterol was assayed each 0.5 h, and means (SE) were calculated (before). At 2 h, 20 mg of a triolein emulsion along with 2 mg rat apolipoprotein E (apo E) was added. Perfusate was assayed 2 h after the addition and at 0.5-h intervals upon concluding the perfusion at 6 h (after). Two mL of phosphate-buffered saline was added to the control perfusion at 2 h. Each data point represents means of four 0.5-h time points. Significance of changes is indicated by $^bP < 0.01$, $^cP < 0.02$, and $^dP < 0.05$.

trols with cholesterol being in the free and esterified form. Incubations of the cholesterol-rich hepatocytes in monolayers with 200 μg of egg phosphatidylcholine/free cholesterol (P/C) in a 1:1 molar ratio supplemented with apo E (100 μg) (P/C-E) revealed no differences in media cholesterol following incubation when compared with the unsupplemented P/C [P/C-E; 2 h, 3.8 (0.2); 14 h, 3.5 (0.1); P/C; 2 h, 3.5 (0.3); 14 h, 4.0 (0.2) μg cholesterol/mg cell protein; n = 4 (SE)].

TABLE 3

Media Cholesterol of Different Cholesterol-Rich Liver Cells After Exposure to Triolein-Rich Emulsions and/or Apo E[a]

Additions	Light hepatocytes	Heavy hepatocytes	Kupffer cells
	(μg media cholesterol/mg cell protein)		
None	3.8 (0.1)	2.8 (0.6)	3.0 (0.8)
Triolein emulsion (200 μg TG/mL)	4.4 (0.6)	2.6 (0.4)	3.0 (0.4)
Triolein emulsion (200 μg TG/mL plus apo E 100 γ/mL)	9.6 (0.2)[b]	6.4 (0.4)[b]	3.9 (0.2)
Apo E 100 μg	3.6 (0.1)	4.4 (0.4)	

[a]The results are the means (SE) of triplicate 12-h MEM incubations after an initial 2-h exposure of the cells to the indicated media additions in MEM. Cultured cells isolated from a Percoll gradient and plated for more than 24 h were incubated in MEM with the additions indicated. After the 2-h incubation, the cells were washed and the medium changed to MEM and assayed for cholesterol after 12 h. The light and heavy hepatocytes had cholesterol contents of 83 (SE 8; n = 10) and 48 (SE 12; n = 8) μg cholesterol/mg cell protein, respectively. Abbreviations as in Table 1.
[b]Significantly greater than any of the other media at $P < 0.001$. The percent distribution of cholesterol in the media high density lipoprotein, low density lipoprotein and very low density lipoprotein of the light hepatocytes following apo E-triolein addition was 34, 38 and 28, and for the light hepatocytes with no addition 46, 24 and 31, respectively.

TABLE 4

Cholesterol Secretion of Hepatocytes in Monolayers Following Exposure to Apoprotein Supplemented Triolein Emulsions[a]

Additions	μg Media cholesterol/mg protein	
	2 h	14 h
None	1.92 (0.07)	2.34 (0.19)
Apo E (50 μg)	2.38 (0.17)[b]	3.21 (0.09)[c]
Apo C-III (50 μg)	1.95 (0.02)	2.45 (0.13)
Apo A-I (50 μg)	1.85 (0.02)	2.59 (0.24)

[a]The data are the means of quadruplicate determinations for each point after an initial 2-h incubation of cholesterol-rich hepatocytes (44 ± 13 μg/mg) with 200 μg of a triolein emulsion and the indicated apolipoproteins. The data are significantly different than the incubation without apoproteins at $^bP < 0.05$ and $^cP < 0.01$.

Addition of 20 mg of triolein emulsion and 2 mg of rat apo E to the perfusate of an isolated perfusion of a cholesterol-rich liver resulted in higher perfusate cholesterol levels (Table 2). The emulsion was cleared within 30 min, and the cholesterol increase became apparent 2–3 h thereafter. A control perfusion did not show such a difference. The perfusate cholesterol was essentially all (>93%) in the d < 1.063 fraction both before and after infusing the apo E-supplemented triacylglycerol emulsion. The percentage of free cholesterol (mean ± SE) was not significantly different before (34 ± 9%, n = 12) and after the addition (37 ± 11%, n = 10).

The cholesterol-loaded hepatocytes were fractionated according to their density into hepatocytes with higher or lower cholesterol contents, and into Kupffer cells. In the 12-h interval following the initial 2-h incubation upon media supplementation, significantly more cholesterol was found in the media of both the lighter and heavier hepatocytes exposed to apo E-triolein emulsions than after any other treatment (Table 3). The hepatocytes containing relatively more cholesterol (lighter cells) showed a more active secretion into the medium than did the heavier cells. The fraction of cell cholesterol secreted by both the less dense and the more dense cells was similar (less dense, 11.4 ± 2.8%; more dense 13.1 ± 3.1%). As was observed in the hepatic perfusions (Fig. 2), most of the medium cholesterol was associated with the VLDL and LDL fractions following exposure of the lighter hepatocytes to the apo E-triolein. Kupffer cells were not responsive to triolein emulsions with or without apo E. The substitution of apo E by apolipoprotein C-III or A-I in the triolein emulsions incubated with the cholesterol-rich cells did not provoke an increase in media cholesterol levels either at the early (2 h) or the late (14 h) time points (Table 4).

DISCUSSION

Rats on cholesterol-supplemented diets acquire appreciable hepatic cholesteryl ester levels within weeks. If the animals are then exposed to the influx of plasma chylomicrons, either following a lipid-rich meal (6) or in-

travenous triacylglycerol injection, they respond with an appreciable increase in plasma cholesterol levels. An increase in plasma apo B and apo E parallels this cholesterol rise, which is predominantly associated with LDLs and VLDLs (7).

Anhepatic cholesterol-fed rats lack this response to either chylomicrons or triglyceride emulsions (6), suggesting that the liver may be the source of increased plasma cholesterol levels. The data presented here are consistent with this. When cholesterol-rich livers are challenged with chylomicrons or triacylglycerol emulsions in the presence of apo E, they respond by secreting lower density cholesterol-rich lipoproteins. This was shown in isolated hepatic perfusions and in cultures of hepatocytes obtained from rats fed 2% cholesterol for at least four weeks to increase the content of cholesteryl esters in the liver.

Hepatic secretion of cholesterol following plasma clearance of particulate triacylglycerols could have several causes. The clearance of chylomicrons or emulsified triacylglycerols invariably (20,21) results in a free fatty acid influx, which could stimulate hepatic VLDL production. However, the fact that intravenous infusion of equal or greater amounts of free fatty acids failed to provoke this response in the cholesterol-fed rat rules out this possibility (6). The LDLs that increase in plasma also do not resemble VLDL (7).

Plasma clearance of particulate triacylglycerols is also followed by the accumulation of residual plasma phosphatidylcholines from the particles. Infusion of phosphatidylcholine into chow-fed rats is known (22) to recruit free cholesterol from tissue, which is then acylated by plasma lecithin: cholesterol acyltransferase. In cholesterol-fed rats with more tissue cholesterol, this process could be accentuated. The inability of phosphatidylcholine liposomes to generate a response similar to that of chylomicrons makes this mechanism unlikely, as does the fact that chylomicron phosphatidylcholine is primarily transferred to HDL and not to LDL.

The lack of a similar plasma cholesterol increase in chow-fed rats upon chylomicron influx re-emphasizes the importance of the hepatic cholesterol pool. This lack of a response in chow-fed rats could either be due to: (i) an inappropriate hepatic stimulus, (ii) an appropriate stimulus with little hepatic response or (iii) both an appropriate stimulus and response but insufficient hepatic cholesterol mass to alter plasma levels. We favor the final explanation for a number of reasons. When triacylglycerols are given to rats on a fat-free diet, an impressive increase in acetate incorporation into cholesterol is observed after a latent phase similar to that seen in the plasma response in cholesterol-fed rats (23). When hepatocytes from chow-fed rats are given labelled cholesterol before being placed in monolayer culture, the cholesterol label can be provoked to appear in the medium, without an increase in cholesterol mass, by exposure to apo E-supplemented triacylglycerol emulsions (manuscript in preparation). These observations suggest that the hepatocytes from chow-fed rats, without stored cholesterol, are affected by the influx of chylomicron remnants but do not generate a sufficient level of lipoprotein cholesterol secretion to produce an increase in plasma cholesterol.

The mechanism by which the apo E-rich emulsions or chylomicrons induce the secretion of cholesterol-rich lipoproteins is unknown. Previous studies (7) have shown an increase in both plasma apo B and apo E with cholesterol, so it is not simply cholesterol enrichment of existing lipoproteins but the formation of new lipoproteins. The plasma cholesterol increase was mainly due to stored hepatic cholesterol with a lesser synthetic component. This may also be the case for the apoprotein B and E increments. The entry of the chylomicron remnant into the liver produces a considerable influx of apo E. This apoprotein may have an endosomal transit different than that of apo B dissociating from the chylomicron lipid, with routing to the golgi similar to transferrin. This apo E cycle may actually provoke secretion of hepatic cholesterol upon exiting the cell. This mechanism is currently under study.

The observation that the clearance of triacylglycerol-rich particles provokes an increase in lower density lipoprotein cholesterol may also be relevant to humans. The conventional Western diet contains significant amounts of cholesterol, and individuals on such a diet have shown a dramatic effect (4) of lipid restriction on plasma LDL cholesterol levels. This may not be totally due to cholesterol intake and LDL receptor activity (3), but may be partly in response to the increase in particulate triacylglycerol clearance occurring on lipid-rich diets. The reciprocal relationship noted for triacylglycerol-rich lipoproteins and LDL may also be attributable to this. An aberration in triglyceride-rich lipoprotein clearance, even as observed in dysbetalipoproteinemia (Type III), may not provide the appropriate stimulus for LDL cholesterol production and may result in lowering LDL cholesterol levels.

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A Cross-Species Comparison of Neutral Lipid Composition of Milk Fat of Prosimian Primates

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The fatty acid composition of milk fat is known to be affected by dietary and genetic differences, while the milk triacylglycerol structure is believed to be attuned to the needs of the subsequent lipolysis during gastrointestinal passage. The availability of milk samples from eight species of prosimian primates, whose milk triacylglycerol structure had not been analyzed, offered an opportunity to further assess these ideas. The milk samples were collected by manual expression and the lipids extracted with chloroform/methanol (2:1, vol/vol). The lipid classes were resolved by thin-layer chromatography, and the neutral lipids subjected to detailed analyses by capillary gas–liquid chromatography of fatty acids and molecular species of triacylglycerols using nonpolar and polarizable liquid phases. The milk samples were found to differ greatly in total fat content (4–73%) and in the composition of the neutral lipid classes and molecular species. The concentration of triacylglycerols ranged from 88–95%, free fatty acids from 0.5–10%, alkyldiacylglycerols from 0.5–5.0%, and diacylglycerols, monoacylglycerols and free and esterified cholesterol made up the remainder. The fatty acid chain length ranged from C_8–C_{24}, with palmitic (16–31%) and oleic (13–40%) acids being the major components in most of the species. In all instances, the molecular association of the fatty acids differed from random distribution by a higher proportion of the monoacid (trioleoyl) and diacid (dipalmitoyloleoyl) glycerols. The phylogenetic influences on neutral milk lipid composition, however, remained unclear, as some of the differences between closely related species were greater than those between more distantly related ones.

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Triacylglycerols (TG) compose 98–99% of the milk fat of most mammals (1). In nonruminants, the TG are largely comprised of long-chain fatty acids of various degrees of unsaturation. About 20 different species of acids are generally present in larger than trace amounts. It has been suggested that the TG fatty acids in milk are largely derived from dietary fat and plasma TG, although the mammary gland is also know to synthesize fatty acids (2–4). The composition and molecular association of the fatty acids in the TG molecules are genetically influenced (1,4). Recent work has suggested (5) that the milk TG structure is tailored for rapid hydrolysis by the concerted actions of lingual and gastrointestinal lipases. The availability of the milk samples from eight species of prosimian primates provided an opportunity to examine the hypothesis further.

TG composition had been previously determined only in the catarrhine primates, such as macaques (6), baboons (7) and humans (8,9), but very little is known about the milk fats of the prosimian primates. Constituent fatty acids have been studied, however, in two closely-related prosimian species (6,10). The present data show that the neutral milk fats of the prosimian species are similar to those of other primates considering their diet and physiological state.

MATERIALS AND METHODS

Animal species studied. Milk samples were collected from eight species of prosimians (Prosimii: Primates) housed at the Duke University Primate Center. These species were: (i) *Eulemur fulvus* (brown lemur); (ii) *E. macaco* (black lemur); (iii) *E. mongoz* (mongoose lemur); (iv) *E. rubriventer* (red-bellied lemur); (v) *Hapalemur griseus* (gentle bamboo lemur); (vi) *Varecia variegata* (ruffed lemur); (vii) *Nycticebus coucang* (slow loris); and (viii) *Otolemur garnettii* (greater bushbaby or greater galago).

Milk collection and storage. Mothers and offspring were separated one to three hours prior to milking to allow accumulation of milk in the glands. Milk samples were obtained by manual expression following intramuscular injection of oxytocin (approximately 4 g/kg) to stimulate milk ejection. Each teat was evacuated as fully as possible, and any discernible contaminants were removed manually before storing at –80°C for subsequent analysis.

An attempt was made to collect samples near mid-lactation when milk composition is likely to be relatively stable. For lemuroids, samples were collected between 40 and 75 d *postpartum*; the sample for *N. coucang* was collected 32 d after parturition. Unfortunately, only an early lactation sample collected four days *postpartum* was available for *O. garnettii* when milk lipid analysis was initially undertaken. However, a subsequent cross-check for free fatty acid content and TG carbon distribution using an *O. garnettii* milk sample collected seventeen days *postpartum* revealed a similar lipid composition. Samples collected from *V. variegata, N. coucanq* and *E. mongoz* were shipped on dry ice for analysis. Some thawing of these samples may have occurred during shipment, as not all samples arrived frozen.

Diets. All animals were fed once daily and provided water *ad libitum* from drip-water bottles. The lemuroid diet consisted of dry biscuits (Purina Monkey Chow 5037; Purina Mills, St. Louis, MO) and various types of cut fruit, except for the diets of *E. mongoz* and *H.*

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Abbreviations: ADG, alkyldiacylglycerol(s); CE, cholesteryl ester(s); GC/MS, gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; L, linoleoyl; La, lauroyl; M, myristoyl; O, oleoyl; P, palmitoyl; Po, palmitoleoyl; S, stearoyl; TG, triacylglycerol(s); TLC, thin-layer chromatography. Triacylglycerol structures are abbreviated by listing their three constituent fatty acids in sequence, e.g., PPP, LaOL.

griseus, which contained less fruit but a variety of fresh vegetables. *Hapalemur griseus* were also provided with bamboo, part of their native diet, and the bamboo leaves were eaten readily by the subjects. Different fruits and vegetables were provided daily.

Otolemur garnettii and *N. coucanq* were both fed cracked high-protein biscuits (Purina Monkey Chow) and fruit. The diet of *O. garnettii* was supplemented once a week with mineral oil-soaked crickets, and a hard-boiled egg once every two weeks. The diet of *N. coucanq* was also regularly supplemented with crickets.

Lipid extraction. Milk samples (0.1–0.5 mL) were lyophilized and then transferred to 1 × 15 mL centrifuge tubes with 1 mL water. The dispersed milk samples were diluted with 3 mL methanol and vortexed, followed by addition of 2 mL chloroform containing 1 mg tridecanoylglycerol as internal standard and vortexing. After addition of 4 mL chloroform and 1.25 mL saline, the tubes were vortexed and centrifuged. The lower chloroform layer was collected and dried by passing through an anhydrous sodium sulfate column and the eluate evaporated under nitrogen. The dry residue was redissolved in a small amount of chloroform and an aliquot of the total lipid extract used for the determination of total lipid profile by capillary gas–liquid chromatography (GLC) with a nonpolar liquid phase (11). The samples were rerun after trimethylsilylation.

Isolation of TG. The TG fraction was recovered from each milk lipid extract by thin-layer chromatography (TLC) on Silica Gel H (Merck, Darmstadt, Germany). The plates (20 × 20 cm, 250 μm thick layer) were developed with hexane/diethyl ether (150:30, vol/vol), which retained free fatty acids close to the origin (R_f = 0.16). The TG were resolved (11) into two bands—a small upper zone (R_f = 0.61–0.75) containing alkyldiacylglycerols (ADG) and a large lower zone (R_f = 0.29–0.61) containing TG. Some milk samples contained a minor band (R_f = 0.88) running ahead of ADG, which was due to cholesteryl esters (CE). The purified neutral lipid bands were recovered from the TLC plates by extraction with chloroform and were saved for further analysis.

Determination of fatty acids. Samples were transmethylated with sodium methoxide (12), neutralized with dilute acetic acid in hexane and extracted with a small portion of chloroform. The organic phase was washed once with water and once with water containing one drop of aqueous ammonia. The lower phase was dried by passing through a short column of anhydrous sodium sulfate. The eluate was directly injected into an SE-54 capillary GLC column using an on-column injector, and the carbon chain length of the fatty acids was determined (12). The remainder of the fatty acid methyl ester sample was brought to dryness and redissolved in hexane. The fatty acid methyl esters were then re-analyzed on a 15-m RTx 2330 polar capillary GLC column (Restek Corp., Port Matilda, PA) using a 7:1 split injection system (13). The carrier gas was hydrogen at 2 psi, and the column temperature was programmed from 80 to 130° at 20°C/min and then to 220°C at 5°C/min (13). The results from nonpolar and polar GLC columns were used together to calculate the final composition.

Determination of TG. The molecular weight distribution of the purified TG and ADG was determined using an 8-m DB-5 (SE-54 equivalent) capillary GLC column (J&W Scientific, Folsom, CA) as described previously (13). The molecular association of fatty acids in the TG and ADG was determined by GLC of the intact molecules on a polarizable capillary column (25 m × 0.25 i.d.) coated with 65% phenylmethylsilicone film (OV-22 equivalent; Quadrex, New Haven, CT) (14) using hydrogen as carrier gas (20 psi). Samples were injected on-column at 40°C and, after elution of the solvent, the oven was ballistically heated to 220°C, when the integration mode was initiated and the column temperature was programmed at 10°C/min to 320°C and then at 2°C/min to 360°C. The data from the GLC analyses on both the nonpolar and polar capillary columns were used for the final calculations.

Determination of ADG. Alkylglycerols and fatty acid methyl esters were prepared from ADG by transmethylation with 1 N sodium methoxide. Alkylglycerol diacetates were prepared with acetic anhydride/pyridine (1:1, vol/vol) and analyzed along with the fatty acid methyl esters by GLC using both nonpolar (SE-54) and polar (SP-2380) capillary columns, as previously described (13). The identities of the peaks were confirmed by gas chromatography/mass spectrometry (GC/MS) using an HP 5989 mass spectrometer and an HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA). Injections were made *via* a splitless injector port set at 290°C and a 12 m × 0.2 mm capillary column coated with 0.33 μm HP-1 (Hewlett-Packard) was used. The oven temperature was initially held at 100°C for 0.5 min and then programmed to 190°C at 20°C/min, to 240°C at 5°C/min, and finally to 290°C at 10°C/min. Electron impact mass spectra (*m/z* 50–500) were acquired every two seconds using 70 eV ionization energy. The alkylglycerol diacetates were identified primarily on the basis of the $[M - 103]^+$, $[M - 60]^+$, $[alkyl]^+$ and *m/z* 159 ions (15).

RESULTS

Figure 1 shows a representative GLC profile of the neutral lipid fractions isolated from milk samples (*E. macaco*). The even carbon numbered TG (C_{38}–C_{54}) constituted the bulk of the neutral lipids. There were also small amounts of odd carbon numbered TG, along with overlapping ADG (see below), which emerged between the even carbon numbered TG and possessed a normal distribution curve (C_{39}–C_{49} on the TG scale). The diacylglycerol peaks were resolved into saturated and unsaturated fractions, which varied in proportion with chain length (C_{26}–C_{36}). The next largest fraction was due to free fatty acids, which ranged from C_8–C_{18}, and also yielded partial resolution between saturated and unsaturated species. Free cholesterol and monoacylglycerols were present in the lowest amounts. Table 1 gives the quantitative composition of the neutral lipid classes of the various milk samples. The fat concentration of the samples varied from 0.29 to 7.3%, consistent with previous analyses employing gravimetric methods (16,17).

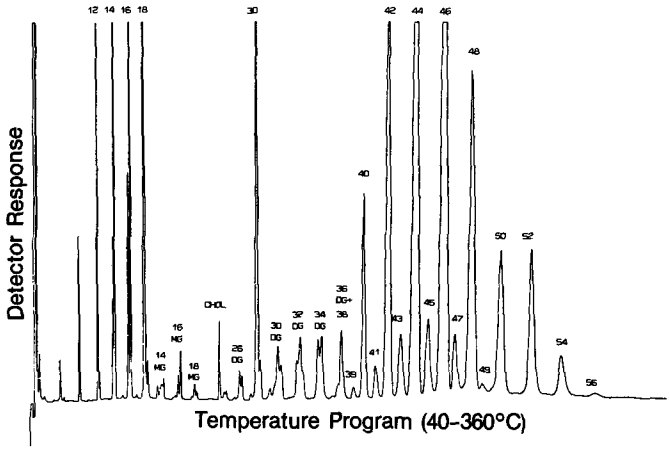

FIG. 1. Total neutral lipid profile of a representative prosimian (*Eulemur macaco*) milk sample as obtained for the trimethylsilyl ethers by capillary gas-liquid chromatography on a nonpolar liquid phase. Peak identification: 8–18, free fatty acids of 8–18 acyl carbons; 14–18, monoacylglycerols (MG) with 14–18 acyl carbons; CHOL, free cholesterol; 26–36, diacylglycerols (DG) with 26–36 acyl carbons; 38–56, triacylglycerols with 38–56 acyl carbons; 39–49, alkyldiacylglycerols with 40–50 carbons in the chains. GLC conditions as described previously (Ref. 12).

Proportionally, the TG made up 70–95% of total lipid. The major nonglycerolipid component was free fatty acid (1–16%) with a nearly constant proportion of free diacylglycerols (3–10%). Monoacylglycerols were absent. On the basis of the molecular weight distribution, the diacylglycerols were largely made up of the 14–16, 16–16 and 16–18 combinations of saturated and unsaturated fatty acids. The peak identity was confirmed by TLC/GLC analysis and by cochromatography with standards. The presence of the ADG was confirmed by a GC/MS identification of the alkylglycerol moieties as the diacetates.

Table 2 gives the carbon number distribution of the neutral lipid classes as obtained by GLC on nonpolar capillary columns for the eight milk samples. The carbon numbers were consistent with the free fatty acid composition, although a random distribution did not appear to be present. The odd carbon numbered TG, ADG, and CE were combined and recorded under the odd carbon number of TG. Separate estimates for the ADG are given below.

Figure 2 compares the carbon number distribution for the TG and ADG purified from a milk sample especially rich in the alkyl ethers (*E. rubriventer*). The ADG were made up of mixed fatty acid esters of C_{16} (28%), C_{18} (48%) and $C_{18:1}$ (23%) alkylglycerols ranging in total nonglycerol carbon number from C_{38} to C_{58}. The ADG were estimated to make up about 0.5% of the total TG fraction in this sample. Other milk fat samples contained less ADG, but possessed similar carbon number distributions.

Table 3 shows considerable variation in the fatty acid composition of the purified TG samples, although nearly all contained 8:0–24:1 acids. Palmitic acid was a major fatty acid, comprising over 20 mole percent in all milks except that of *E. fulvus*, which contained myristic and lauric as the major fatty acids. *Varecia variegata, O. garnettii* and *E. mongoz* contained oleic acid as the major component. All samples contained significant amounts (7–14%) of linoleic acid, but only traces of 20:4 and other long chain polyunsaturated fatty acids. The fatty acids of the purified ADG fractions contained, on average, 14:0 (22%), 16:0 (30%), 18:1n-9 (17%) and 18:2 (11%) as major components and 12:0 (7%), 16:1 (8%), 18:0 (3%) and 18:1n-7 (1.4%) as minor components.

Figure 3 gives the polar capillary GLC profiles of intact TG from two milk samples from *E. rubriventer* (Fig. 3A) and *V. variegata* (Fig. 3B), which differed greatly in

TABLE 1

Neutral Lipid Class Composition of Some Prosimian Primates[a]

Lipid class[b]	Primate species							
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	(mg/g fresh weight)							
Total	4.0	11.5	2.9	—	29.4	9.5	10.2	73.1
Total[c]	5.0	11.5	3.1	10.4	29.1	13.9	16.3	85.5
	(percent total)							
CHOL[c]	0.7	0.4	0.8	0.4	0.4	0.5	0.3	0.4
FFA	16.5	11.4	12.2	6.8	11.2	7.1	trace	1.3
DG	10.1	7.0	4.7	7.1	9.3	5.4	trace	3.2
ADG + CE	2.7[d]	6.0	0.1	2.3	1.7	4.1	7.2	0.0
TG	70.1	75.2	82.4	82.4	77.4	82.9	92.5	95.2
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]Primate species: (1) *Eulemur fulvus;* (2) *E. macaco;* (3) *E. mongoz;* (4) *E. rubriventer;* (5) *Hapalemur griseus;* (6) *Varecia variegata;* (7) *Nycticebus coucang;* (8) *Otolemur garnettii.*

[b]CHOL, free cholesterol; FFA, free fatty acids; DG, diacylglycerols; ADG + CE, alkyldiacylglycerols + cholesteryl esters; TG, triacylglycerols.

[c]Single estimates based on species mean dry matter values.

[d]Single estimates; values subtracted from total lipid profiles.

TABLE 2

Carbon Number Distribution of Neutral Milk Lipids of Some Prosimian Primates (% lipid class)[a]

Lipid class	Primate species (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Free fatty acids								
C_8	1.5	1.3	1.1	2.5	1.1	2.1	1.0	
C_{10}	10.2	4.3	1.2	11.7	15.9	2.1	5.2	5.0
C_{12}	21.9	13.8	3.3	17.4	18.1	5.9	5.5	6.3
C_{14}	20.4	20.8	6.4	16.1	12.5	9.6	7.5	7.7
C_{16}	15.8	25.3	25.7	18.3	20.3	22.5	23.6	26.6
C_{18}	30.3	34.6	62.3	34.0	32.2	57.9	57.2	54.4
Diacylglycerols								
C_{26}	7.9			4.7	3.6		2.3	
C_{28}	16.5	7.1	3.5	10.2	8.0		5.3	3.5
C_{30}	17.2	10.9	12.2	12.8	9.0	2.8	11.4	4.3
C_{32}	13.5	20.6	11.1	14.9	12.7	9.9	13.4	11.6
C_{34}	22.4	23.5	31.7	22.8	23.5	23.4	21.3	24.1
C_{36}	17.3	25.8	34.9	26.5	32.1	44.8	33.7	34.6
C_{38}	5.2	12.1	6.6	8.1	10.0	19.1	12.7	21.9
Cholesteryl esters + alkyldiacylglycerols + triacylglycerols[b]								
C_{37}	0.0	0.0	0.04	0.0	0.04	0.0	0.0	0.04
C_{39}	0.1	0.0	0.0	0.1	0.1	0.04	0.0	0.1
C_{41}	0.1	0.1	0.0	0.2	0.2	0.1	0.1	0.2
C_{43}	0.3	0.4	0.1	0.3	0.5	0.2	0.2	0.4
C_{45}	0.3	0.5	0.2	0.4	0.5	0.4	0.5	0.5
C_{47}	0.2	0.5	0.3	0.3	0.5	0.5	0.7	0.6
C_{49}	0.1	0.3	0.5	0.2	0.5	0.7	0.9	1.1
C_{51}	0.0	0.3	0.9	0.2	0.5	0.8	1.1	1.8
C_{53}	0.0	0.1	0.5	0.0	0.3	0.6	0.8	1.3
C_{55}	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.3
Triacylglycerols[b]								
C_{32}	0.3	0.1	0.0	0.2	0.2	0.0	0.2	0.2
C_{34}	1.1	0.1	0.0	0.6	0.6	0.0	0.2	0.5
C_{36}	3.2	0.4	0.1	1.4	1.4	0.1	0.5	1.0
C_{38}	7.7	1.4	0.4	3.4	3.2	0.4	1.0	2.1
C_{40}	14.3	5.2	1.0	7.6	6.6	1.1	2.0	3.2
C_{42}	18.6	14.0	1.9	15.1	12.6	3.4	4.5	4.7
C_{44}	22.0	23.7	3.8	23.0	18.2	8.2	9.2	6.8
C_{46}	15.6	24.7	5.9	20.7	15.6	12.4	15.4	7.8
C_{48}	6.7	13.8	9.8	10.2	11.1	12.5	16.6	9.6
C_{50}	4.1	6.5	21.0	6.3	11.9	19.3	15.7	16.4
C_{52}	4.2	5.6	40.0	5.5	11.7	28.7	21.3	26.2
C_{54}	1.3	2.0	11.2	2.0	3.6	9.6	7.1	12.4
C_{56}	0.0	0.5	2.2	1.9	0.2	0.9	1.2	2.2
C_{58}	0.0	0.0	0.4	0.5	0.0	0.0	0.4	0.5
C_{60}	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1

[a]Primate species as in Table 1. Single determinations from a series of calibrated runs.
[b]As percentage of combined cholesteryl esters, alkyldiacylglycerols and odd and even carbon number triacylglycerols.

the average carbon number and degree of unsaturation. Figure 3A shows the profile obtained for the shorter chain TG sample. The molecular species ranged from C_{32} to C_{54}. Only the longer chain species could be tentatively identified, the short chain species showed more overlap and probably gave resolution of isobaric TG within carbon and double bond numbers, as previously observed for other short chain TG (14). Figure 3B shows the profile obtained for the longer chain TG sample. The molecular species ranged from C_{32} to C_{54} with extensive

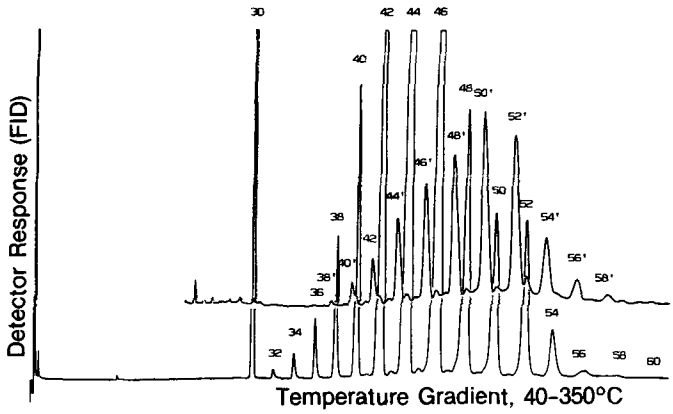

FIG. 2. Carbon number distribution in the triacylglycerol and alkyldiacylglycerol fractions purified from the same sample of milk (*Eulemur rubriventer*). Peak identification of triacylglycerols and alkyldiacylglycerols and abbreviations as in Figure 1. GLC conditions as given in text.

resolution based on the number of double bonds per TG molecule within each carbon number. While the identity of the long chain TG could be readily established (14,18), that of the short chain TG could be determined only with

TABLE 3

Fatty Acid Composition of Triacylglycerols Purified from the Milk Samples of Some Prosimian Primates (mole %)[a]

Fatty acids	Primate species (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
8:0	0.2	0.2	0.0	1.0	1.6	0.0	0.0	0.6
10:0	7.6	3.4	1.1	8.5	8.2	1.7	0.7	3.8
10:1?	0.1	0.0	0.0	0.1	0.2	0.0	0.0	0.0
12:0	22.5	15.6	2.9	15.4	9.6	5.6	7.5	5.8
12:1?	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0
14:0	21.2	19.5	6.2	16.6	11.4	9.0	12.1	7.3
14:1	2.4	1.8	0.4	1.5	0.6	0.5	0.1	0.3
15:0	0.0	0.1	0.1	0.1	0.2	0.1	0.3	0.2
15:1	0.0		0.0	0.0	0.0	0.0	0.1	0.2
16:0	16.1	28.0	27.4	20.6	22.0	24.9	31.2	22.6
16:1n-7	4.0	5.3	6.8	4.5	6.5	6.4	2.5	5.9
17:0	0.0	0.0	0.2	0.2	0.2	0.2	0.6	0.4
17:1	0.5	0.5	0.4	0.4	0.6	0.5	0.5	0.9
18:0	1.3	1.5	2.2	2.0	1.4	2.3	4.5	2.6
18:1n-9	13.9	13.0	32.4	14.5	21.0	39.3	22.1	32.9
18:1n-7	1.0	1.7	1.5	1.5	2.0	2.4	3.0	2.4
18:2n-6	7.3	7.2	14.1	9.9	8.7	5.4	12.3	9.2
18:3n-3	0.4	0.4	0.8	0.6	1.0	0.3	0.6	0.8
20:1n-9	0.1	0.2	0.2	0.2	0.2	0.3	0.5	0.6
20:1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2
20:2n-6	0.2	0.3	0.4	0.5	0.2	0.2	0.7	0.4
20:3n-6	0.3	0.3	0.3	0.5	0.3	0.1	0.1	0.5
20:4n-6	0.2	0.5	0.4	0.5	0.4	0.1	0.1	0.9
20:5n-3	0.1	0.1	0.4	0.2	0.1	0.2	0.1	0.1
22:1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1
22:5n-3	0.1	0.2	0.4	0.3	2.4	0.2	0.0	0.4
22:6n-3	0.1	0.2	0.8	0.4	1.0	0.1	0.2	0.5
24:1n-9	0.0	0.0	0.5	0.1	0.3	0.2	0.1	0.4

[a]Primate species as in Table 1. Single determinations from a series of calibrated runs.

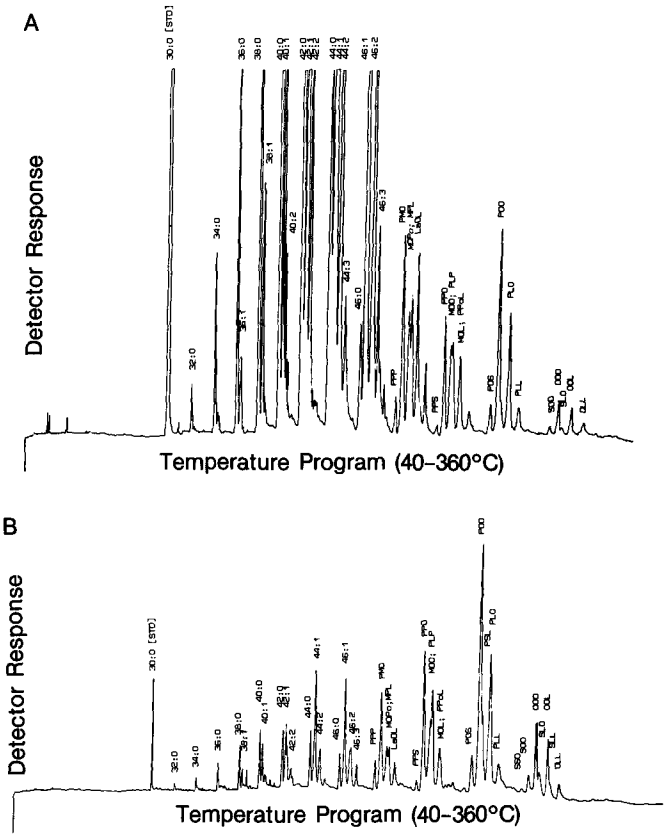

FIG. 3. Polarizable capillary gas–liquid chromatography (GLC) profiles of two prosimian milk samples exhibiting extremes in carbon number distribution. A, *Eulemur rubriventer*; B, *Varecia variegata*. Peak identification as given in the figure and in text; 30:0, tridecanoylglycerol internal standard; 32:0 to 46:3, triacylglycerols with a total of 32–46 acyl carbons and 0–3 double bonds. P, palmitic; M, myristic; S, stearic; O, oleic; L, linoleic acids. GLC conditions were as given in text for the polarizable column GLC of lipids.

respect to the carbon number and degree of unsaturation. Similar patterns were recorded for the TG prepared from the other milk samples. Table 4 gives the quantitative molecular species composition of the milk TG as calculated by utilizing the results obtained on polar and nonpolar capillary columns. The molecular species of the TG reflected the composition of the major fatty acids, but differed from a random distribution by a higher proportion of monoacid (trioleoyl) and diacid (dipalmitoyloleoyl) TG than calculated on basis of random distribution.

Figure 4 illustrates the polarizable capillary GLC resolution of the ADG isolated from one of the milk samples (*E. rubriventer*). The peaks were identified by reference to the fatty acid and alkylglycerol compositions, the distribution of the carbon numbers obtained by nonpolar capillary GLC, and by reference to standards. There was one saturated (both medium and long chain) or one monounsaturated chain per molecule. Table 5 gives the quantitative composition for two of the ADG samples resolved on the polarizable capillary GLC column. The proportions of the various ADG reflected the fatty acid and alkylglycerol compositions, but differed significantly from the distributions anticipated from a strictly random combination of the respective chains.

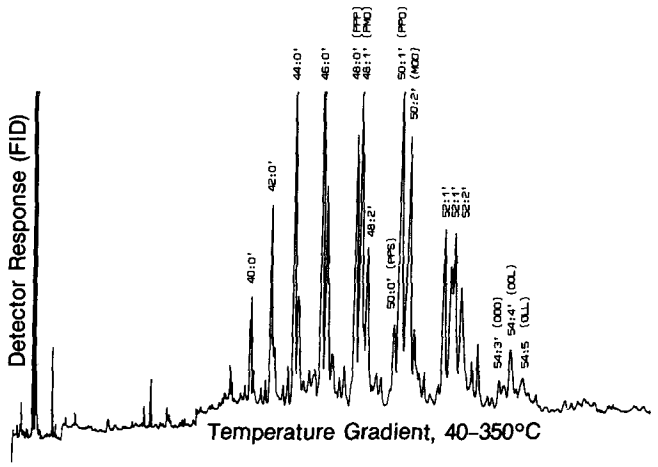

FIG. 4. Polarizable capillary GLC profile of the purified alkyldiacylglycerol fraction from a prosimian milk sample (*Eulemur rubriventer*). Peak identification and abbreviations as in Figure 3. GLC conditions as in text and in Figure 3.

DISCUSSION

The availability of milk samples from eight species of prosimian primates, which previously had not been analyzed, offered an opportunity to examine the validity of some current hypotheses about the purposeful design of milk fat TG structure. *Eulemur* species, *Varecia* and *Hapelemur* are members of a monophyletic group, the Lemuroidea, who range in size from approximately one to three kilograms, and are only found today on the island of Madagascar. Recent work has confirmed the close relationships among the separate *Eulemur* species and suggested that the *Hapalemur* species, along with *Lemur catta*, form a sister group to *Eulemur* (19–23). The relationship of *Varecia* to the other lemurs is more distant still. Recently, the inclusion of *Varecia* with *Eulemur*, *Lemur* and *Hapalemur* in the family Lemuridae has been questioned (Refs. 20, 22; J.M. Macedonia and K.F. Stanger, 1993, unpublished results).

Nycticebus coucang and *O. garnettii* are representatives of two major sister taxa within the suborder Lorisidea. The slow loris, *N. coucang*, represents the Lorisoidea (lorises), and *O. garnettii* represents the Galagidae (galagos). Lorisoids are continentally-distributed, small (0.2–1.2 kg), nocturnal prosimians. Lorises are found in both Africa and Asia; the slow loris, *N. coucang*, is widely distributed throughout southeast Asia. Galagos, including *O. garnettii*, are found on the African continent.

Fatty acid composition. In common with many other mammalian species, the studied prosimians produced milk fat containing large proportions of palmitic and oleic acids. Short chain fatty acids (less than C_8) were absent. The odd carbon numbered fatty acids, which constitute a significant proportion of total fatty acids in ruminant milk fats (13), were also largely absent. Fatty acid chains of C_8 were present in significant amounts only in *E. rubriventer* and *H. griseus*. Milks of *Eulemur* species generally contained high levels of medium chain (C_{10} to C_{15}) acids (40.5–54.3%), except for *E. mongoz*, which had the lowest (10.5%). The milk fats of *E. fulvus*,

TABLE 4

Molecular Species Composition of Milk Triacylglycerols from Some Prosimian Primates (area %)[a]

Molecular species[b]	Primate species (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Molecular species[b]	Primate species (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
32:0	0.2	0.1	0.0	0.2	0.2	0.0	0.1	0.2	48:0 (PPP)	0.3	1.2	1.0	0.7	1.0	3.7	5.1	1.2
									48:1 (PMO)	1.7	5.5	5.0	2.9	3.7	5.5	6.4	4.0
34:0	0.9	0.1	0.0	0.5	0.4	0.0	0.2	0.5	48:2 (MPoO)	1.1	4.8	0.0	0.0	1.6	1.5	4.4	1.8
									48:2 (MPL)	1.2	0.0	3.3	3.7	2.3	1.3	0.0	1.8
36:0	2.6	0.3	0.1	1.0	0.8	0.1	0.5	0.9	48:3 (LaOL)	1.8	1.6	1.0	2.0	2.2	0.7	1.6	1.8
	0.5	0.1	0.0	0.3	0.4	0.0	0.1	0.0		0.7	0.7	0.0	0.7	0.8	0.4	0.0	0.0
	0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.2		0.0	0.4	0.0	0.5	0.0	0.1	0.0	0.0
38:0	5.8	1.0	0.4	2.3	2.0	0.3	0.8	1.1	50:0 (PPS)	0.1	0.3	0.7	0.3	0.6	1.9	2.3	0.9
38:1	1.6	0.3	0.0	0.8	1.0	0.1	0.2	0.5	50:1 (PPO)	1.0	1.5	6.9	1.1	3.0	10.2	5.3	5.8
	0.3	0.1	0.0	0.3	0.0	0.0	0.1	0.5	50:2 (MOO)	0.8	0.7	0.0	1.0	5.1	3.0	0.0	2.4
	0.1	0.0	0.0	0.0	0.2	0.0	0.0	0.0	50:2 (PLP)	0.9	1.8	10.0	1.3	0.0	3.3	5.3	5.3
									50:3 (MOL)	1.0	1.9	3.3	1.9	2.9	1.5	2.6	3.2
40:0	9.0	3.9	0.7	4.7	3.1	1.0	1.6	1.3		0.3	0.7	1.0	1.0	0.8	0.3	1.4	1.6
40:1	3.9	1.2	0.3	2.1	2.1	0.1	0.0	1.0									
	1.0	0.0	0.0	0.5	1.1	0.0	0.3	0.6	52:0 (PSS)	0.0	0.0	0.2	0.0	0.0	1.1	1.2	0.2
	0.2	0.2	0.0	0.2	0.4	0.1	0.2	0.3	52:1 (POS)	0.4	0.3	2.7	0.4	0.8	4.8	2.5	2.3
	0.1	0.0	0.0	0.0	0.1	0.3	0.0	0.3	52:2 (POO)	2.1	1.8	16.6	1.3	3.8	16.1	6.2	11.0
	0.2				0.2				52:2 (PLS)	0.0	0.4	0.0	0.6	1.2	0.0	2.2	3.0
									52:3 (PLO)	1.4	2.2	15.7	2.1	4.3	6.0	7.2	8.1
42:0	7.5	8.5	1.1	6.3	3.8	2.9	2.4	1.7	52:4 (PLL)	0.4	1.0	5.4	1.1	1.9	1.3	2.9	3.0
42:1	7.3	3.5	0.6	5.8	3.2	0.5	0.6	1.4									
	0.0	0.0	0.0	0.0	2.3	0.0	0.6	1.0	54:1 (OSS)	0.0	0.0	0.2	0.0	0.0	0.2	0.6	0.2
	3.2	1.0	0.2	2.5	2.1	0.1	0.6	0.7	54:2 (SOO)	0.2	0.1	1.3	0.1	0.2	0.9	0.9	1.7
	0.2	0.0	0.0	0.2	0.3	0.1	0.3	0.4	54:3 (OOO)	0.4	0.4	2.8	0.3	0.8	4.7	1.2	3.8
									54:3 (SLO)	0.1	0.3	1.7	0.5	0.3	1.1	1.4	1.9
43:0	0.4	0.4	0.0	0.3	0.4	0.0	0.0	0.0	54:4 (OOL)	0.4	0.6	3.6	0.7	1.5	2.3	1.2	3.9
	0.2	0.6	0.0	0.3	0.7	0.0	0.2	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0
	0.2	0.5	0.0	0.0	0.2	0.0	0.0	0.0	54:5 (OLL)	0.3	0.6	1.6	0.5	0.8	0.4	1.1	1.2
44:0	3.5	11.1	0.9	4.8	2.9	6.3	5.0	1.2	C_{56}	0.0	0.5	2.2	1.9	0.2	0.9	1.2	2.2
44:1	10.6	8.9	2.0	10.2	8.9	1.7	2.4	3.0									
44:2	6.6	3.3	0.9	6.2	4.6	0.4	1.3	1.6	C_{58}	0.0	0.0	0.4	0.5	0.0	0.0	0.4	0.5
	1.4	0.4	0.1	1.6	1.2	0.2	0.5	0.6									
	0.0	0.0	0.0	0.0	0.8	0.1	0.3	0.0	C_{60}	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
	0.1	0.6	0.0	0.5	0.3	0.0	0.2	0.9									
46:0	1.0	5.5	0.9	2.1	1.5	7.0	5.4	1.2									
46:1	7.0	11.6	3.2	8.7	6.7	5.0	4.4	3.7									
46:2	5.7	6.1	1.6	7.0	4.7	0.1	2.9	2.4									
46:3	1.1	0.9	0.3	1.5	2.0	0.4	1.2	1.2									
	0.6	0.7	0.0	1.0	0.0	0.0	1.2	0.0									
	0.3	0.3	0.0	0.6	0.6	0.3	1.0	0.0									
	0.0	0.2	0.0	0.0	0.6	0.1	0.0	0.0									

[a]Primate species as given in Table 1. Single determinations from a series of calibrated runs.

[b]Total number of acyl carbons:total number of double bonds in a triacylglycerol molecule; L, linoleoyl; La, lauroyl; M, myristoyl; O, oleoyl; P, palmitoyl; Po, palmitoleoyl; S, stearoyl; C_{56}, C_{58} and C_{60}, triacylglycerols of 56, 58 and 60 acyl carbons, respectively, and unspecified degree of unsaturation.

E. macaco and *E. rubriventer* were very similar to each other and were characterized by a high proportion of C_{12} and C_{14} acids. *Eulemur fulvus* represented the extreme, having a higher percentage of medium chain acids than of C_{16} and C_{18} fatty acids. *Eulemur mongoz* had moderate levels of medium chain acids and greater quantities of C_{18} acids, as did the lorisoid primates and, to some extent, *H. griseus* and *Varecia*. Palmitic acid was prominent in milks of most species, but was particularly high in *N. coucang*. In many respects, the milk of *E. mongoz* resembles that of *H. sapiens*, with similar levels of palmitic, oleic and linoleic acids and smaller quantities of longer chain fatty acids, such as 20, 22 and 24 acyl carbon homologues.

To some extent the differences in fatty acid composition observed among species may be a consequence of dietary differences. The diet of the lactating mother has been shown among human subjects to have a

TABLE 5

Molecular Species Composition of Alkyldiacylglycerols from the Milk of Two Prosimian Primates (area %)[a]

Molecular species	Primate species (4)	Primate species (6)	Molecular species	Primate species (4)	Primate species (6)
C_{38}	0.5	0.1	50:0 (PPS)	1.9	26.1
	0.2		50:1 (PPO)	8.2	
			50:2 (MOO)	7.4	
C_{40}	0.7	0.2	50:2 (PLP)	1.8	
	0.9		50:3 (MOL)	0.7	
	0.5			0.7	
	0.3			0.4	
C_{41}	0.4				
	0.4		52:0 (PSS)	0.6	37.8
			52:1 (POS)	4.2	
C_{42}	2.7	0.7	52:2 (POO)	3.3	
	0.9		52:2 (PLS)	3.7	
			52:3 (PLO)	4.0	
C_{43}	0.3		52:4 (PLL)	1.1	
	0.7			1.4	
				0.3	
C_{44}	5.3	2.0			
	2.5		54:0	0.3	10.9
	0.6			0.8	
				0.8	
C_{45}	0.9			1.4	
	0.6			0.5	
				1.1	
46:0	7.4	5.5		0.4	
46:1	3.8			0.2	
46:2	1.3			0.3	
				0.1	
47:0	1.3			0.1	
	0.3			0.1	
			56:0	0.0	3.2
	0.5			0.1	
	1.0			0.2	
				0.2	
48:0 (PPP)	5.6	13.6		0.2	
48:1 (PMO)	7.8			0.1	
48:2 (MPL)	0.0			0.3	
	3.5			0.2	
48:3 (LaOL)	0.3			0.2	
49:0 ?	0.9				
	0.8				
SUM	99.7			100.0	

[a](4) *Eulemur rubriventer*; (6) *Vareca variegata*; abbreviations as in Table 4. Single determinations from a series of calibrated runs.

predictable relationship with milk composition (4). Human mothers on high-carbohydrate/low-fat diets have been reported to produce milk enriched in medium chain fatty acids (4), which appear to be synthesized *de novo* in the mammary gland rather than being derived from blood or dietary precursors (24). Thus, the high levels of medium chain fatty acids in most *Eulemur* spp. (except *E. mongoz*) may reflect low-fat intakes and relatively increased mammary synthesis. It is possible that the inclusion of vegetables in the diet of *E. mongoz* (not offered to other *Eulemur* species) may partly explain higher levels of long chain fatty acids in the milk of this species as compared to other *Eulemur* species. However, *Varecia* consumes a diet that is comparable to that of the other *Eulemur*, but produces milk more similar in fatty acid composition to that of *E. mongoz*.

The relatively high proportion of free fatty acids in some of the milk samples is reminiscent of that seen in the milk of a human Type I hyperlipoproteinemia patient (11,25), who presumably synthesize much of the fatty acid fraction in the mammary gland due to an inability to clear plasma TG because of a lack of lipopro-

tein lipase (26). Alternatively, the free fatty acids could have come from lipolysis of the milk TG, to which they correspond in composition. However, those samples (*V. variegata, N. coucang* and *E. mongoz*), which had thawed, did not have higher free fatty acid concentration than samples that remained frozen at -80°C prior to analysis.

TG composition and structure. The milk TG of *Eulemur* species tended to have the lowest average acyl carbon number. *Eulemur fulvus* was extreme among the lemuroids, as it had much greater proportions of C_{36} and C_{38} TG in relation to C_{40} to C_{46} TG. *Varecia* species differed from others by having very little C_{40} and little C_{42}. *Eulemur* species (excluding *E. mongoz*) had little or no TG with C_{50} or higher acyl carbons, while the C_{52} TG made up about 40% of the total in *E. mongoz* and 30% in *V. variegata*. Among lorisoids, TG with the longer chains were more prominent, as would be expected from the fatty acid composition. Thus, C_{44}–C_{52} TG were major components of both *N. coucang* and *O. garnettii*. The longer chain TG in all instances were made up mainly of PMO, MPoO, PPO, PLP, POO, PLO, OOO and OOL TG in varying proportions.

The work of Hansen *et al.* (27) has shown that the monoacylglycerol pathway of TG synthesis is absent from the mammary gland of the goat, in which case TG have to be formed exclusively *via* the phosphatidic acid pathway. This pathway proceeds *via* long chain diacylglycerol intermediates with mono- and diunsaturated fatty acids in the *sn*-2 position. Because medium chain fatty acids (C_8–C_{12}) are not readily incorporated into the phosphatidic acid and thus into the diacylglycerol intermediates, it would be anticipated that the short and medium chain fatty acids would occupy mainly the *sn*-3 position, as has already been shown for human (9) and ruminant (28–30) milk fats. In such a case, the biosynthetic diacylglycerol intermediates isolated from the milk fat would contain only long chain species, mainly C_{32}, C_{34} and C_{36} derivatives. As the neutral lipid profiles indicated the presence of both medium and long chain length diacylglycerols (Table 2), as well as small amounts of long chain monoacylglycerols, it would appear that the diacylglycerols were predominantly formed by hydrolysis due to lipoprotein lipase present in fresh milks. This enzyme attacks the *sn*-1 position, preferentially producing medium and long chain diacylglycerols of the *sn*-2,3 configuration (31,32). Alternatively, tissue damage during manual expression of the milk may have led to release of lipases, resulting in lipolysis.

ADG composition and structure. ADG have been detected in milk samples of ruminants (33,34) and in a healthy human female (34). Although the origin of the alkylglycerol moiety of these glycerolipids is not known, dietary alkylglycerols would appear to be the most likely precursors. The *sn*-1 monoalkylglycerols released from food glycerophospholipids in the intestinal lumen are readily absorbed and incorporated into tissue lipids (35,36). Therefore, the alkyl group in the ADG would be anticipated to be located in the *sn*-1 position.

Although the prosimian primates were not maintained on rigidly controlled dietary regimens, the differences in the fatty acid and neutral glycerolipid composition were consistent with dietary influences within the limits of the experimental controls. The phylogenetic influence on neutral milk lipid composition, however, remained unclear as some of the differences between closely related species were greater than those between more distantly related ones.

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Sterol and Fatty Acid Composition of Neutral Lipids of *Paratenuisentis ambiguus* and Its Host Eel[1]

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The sterol composition of free sterol and steryl ester fractions of the fish parasite *Paratenuisentis ambiguus* was determined. In addition, the fatty acid composition of various neutral lipid classes, i.e., wax esters, steryl esters, triacylglycerols and free fatty acids, as well as the composition of the 1-*O*-alkyl moieties of total ether glycerolipids of the parasite, were investigated. The results of these studies were compared with those obtained on the intestinal tract tissue of its host, the eel (*Anguilla anguilla*). Cholesterol is the major sterol in both *P. ambiguus* and *A. anguilla*. However, the sterols of *P. ambiguus* contain high proportions (>20%) of other sterols, such as campesterol and various dehydrosterols. [e.g., 7-dehydrocholesterol and cholesta-5,22(*E*)-dienol]. The presence of these minor sterols agrees with the known biotransformations of exogenous sterols in various helminths. Considerable differences are found in the fatty acid composition of neutral lipid fractions, as well as the total lipid extract from the endoparasite as compared to the host tissue. In particular, eicosapentaenoic acid (20:5n-3), other polyunsaturated fatty acids, such as 20:4n-6, 22:5n-3 and 22:6n-3, as well as long-chain saturated fatty acids, such as 20:0, are generally enriched in the neutral lipid fractions of the parasite as compared to those of infected eel intestine. The analysis of ether glycerolipids revealed that 1-*O*-hexadecyl (16:0) and 1-*O*-hexadecenyl (16:1) moieties were present in similar proportions in the ether lipids of both *P. ambiguus* and eel intestine, whereas 1-*O*-octadecyl (18:0) moieties are more prominent in the parasite and 1-*O*-octadecenyl (18:1) moieties in the eel. The results of these studies show that *P. ambiguus* has specific mechanisms for the regulation of the sterol and fatty acid composition of its neutral lipids.

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Lipid composition and metabolism have not been extensively studied in Acanthocephala. Some earlier histochemical studies have shown that lipid deposits occur in various tissues of these endoparasites (1). The total lipid content, as well as the ratio of neutral lipids to phospholipids, was found to vary among some acanthocephalans, e.g., *Moniliformis moniliformis, Macracanthorhynchus hirudinaceus* and *Sphaerirostris pinguis* (2,3). In addition, differences in total lipid contents have been shown between male and female organisms of the same species (2). On the other hand, the composition and biosynthesis of lipids in various species of fish are now established (4,5). In fish, dietary lipids play an important role as a source of essential fatty acids (EFA) such as 18:2n-6 or 18:3n-3, and of long-chain polyunsaturated fatty acids, i.e., 20:5n-3 and 22:6n-3 (6). The extent to which the acyl moieties are altered by dietary lipids is, however, different in different lipid classes. Generally, neutral lipids of fish, such as triacylglycerols, are affected by feeding an EFA-deficient diet to a larger extent than are phospholipids, whereas the latter are changed preferentially by EFA (6). Little is known about the lipid content and the fatty acid composition of whole intestinal tissue of fish and specifically in the case of the North Atlantic eel, *Anguilla anguilla* (5).

Paratenuisentis ambiguus (Van Cleave, 1921) Bullock et Samuel 1975 (Eoacanthocephala, Tenuisentidae) is an endoparasite found in the intestine of the American (*A. rostrata*) and the European eel (*A. anguilla*) (7,8). Taraschewski (9) observed that the worm's presoma, which is surrounded by intestinal tissue of the eel, apparently collects host lipids at its surface. On the other hand, an osmiophilic substance seemed to be excreted by the parasite through perforations of its proboscis hooks (9). In addition, it was shown that lipids such as tritiated triolein can be absorbed by *P. ambiguus* and other acanthocephalans through the tegument of the presoma (10). Little information is available on the composition and biochemistry of lipids in Acanthocephala, including *P. ambiguus* (1).

There is increasing interest in fish parasitology because of the increasing use of commercial aquaculture and because of the significant financial losses that can be caused by the pathogenic effects of parasitic worms. This prompted us to study the lipids of *P. ambiguus* in comparison with the lipids of infected tissue of eel intestine. Endoparasites are known to have only a very limited lipid metabolism (11). Thus, the aim of our studies was to find differences in the lipid composition between parasite and host that could be utilized to design means to control acanthocephalans and other parasites of eel.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography (HPLC)-grade solvents (acetonitrile, acetone) were purchased from E. Merck (Darmstadt, Germany). Fatty acid methyl ester standards were from Nu-Chek-Prep (Elysian, MN); the fish oil standards "PUFA 1" and "PUFA 3" were from Matreya (Pleasant Gap, PA); "CPL Fish Oil 30" was from Larodan (Malmö, Sweden). Acetylations were carried out in acetic anhydride/pyridine (1:1, vol/vol) at 80°C for 1 h. Methyl esters derived from acyl lipids were prepared by transmethylation using

[1]Dedicated to Professor Helmut K. Mangold on the occasion of his 70th birthday.

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Abbreviations: EFA, essential fatty acids; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; RRT, relative retention time; TLC, thin-layer chromatography.

methanol/benzene/conc. sulfuric acid (84:10:4, by vol); free fatty acids were esterified with diazomethane (12).

Biological materials. Eels (*A. anguilla*) from the Weser river that were naturally infected with *P. ambiguus* were used for the analyses; they were free of other intestinal helminths. The eels were killed and dissected immediately. Intestinal tissues were excised and the attached parasites removed. The parasites were kept overnight at 7°C in minimum essential medium (MEM; Flow Laboratories, Meckenheim, Germany) supplemented with glucose (1 g/L) as well as streptomycin (100 µg/mL) and penicillin (100 U/mL). In addition, sections of infected intestinal tissue were excised from different parts of the anterior eel gut, pooled and then deep-frozen. Both *P. ambiguus* (whole organisms, males and females combined) and eel intestine were used for lipid extraction.

Extraction procedures. Paratenuisentis ambiguus, 5.7 g worm tissue (average weight 2.5 mg/animal) and *A. anguilla*, 6.1 g intestinal tissue, were homogenized in 4 mL of dichloro-methane/methanol (1:2, vol/vol) per gram fresh weight using an Ultra-Turrax blender (IKA-Werke, Staufen, Germany). After centrifugation, pellets were reextracted twice with 10 mL, each, of dichloromethane/methanol (2:1, vol/vol). The combined extracts were diluted with dichloromethane and water, and the phases were separated by centrifugation (13,14). The organic phases were dried and concentrated, and the total lipids of both tissues determined gravimetrically.

Lipid analysis. Aliquots of total lipids were fractionated by preparative thin-layer chromatography (TLC) on Silica Gel H (E. Merck) with hexane/diethyl ether/acetic acid (80:20:1, by vol) as solvent. Fractions containing wax esters plus steryl esters, triacylglycerols, free fatty acids and sterols were isolated and extracted from silica gel using water-saturated diethyl ether. The fractions of polar lipids at the origin of the plates were isolated and extracted with dichloromethane/methanol/water (1:2:0.8, by vol) (13). The polar lipid extracts obtained as described were kept in dichloromethane/methanol (2:1, vol/vol) for further analyses. The fractions consisting of wax esters plus steryl esters were resolved on layers of magnesium oxide with hexane/diethyl ether/ethyl acetate (75:25:1, by vol) as solvent (15). Aliquots of steryl ester fractions were subjected to alkaline hydrolysis (16). The liberated sterols were extracted from the reaction mixture and purified by TLC on Silica Gel H using hexane/diethyl ether (3:2, vol/vol) as solvents. The fractions of free sterols, as well as sterols derived from steryl esters by alkaline hydrolysis, were acetylated for gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) as described previously. All lipid fractions on silica plates were detected by charring after spraying with chromic sulfuric acid and heating at 200°C; fractions were identified by co-chromatography with standards. Lipid fractions on magnesium oxide plates were detected by exposure to iodine vapors. All lipids were quantified by weighing. Aliquots of total lipids as well as of acyl lipid fractions were converted to the methyl esters by methanolysis as described. Fatty acid methyl esters and steryl acetates were purified by TLC on Silica Gel H with hexane/diethyl ether (4:1, vol/vol) as developing solvent, isolated and then analyzed by GC as described next.

Aliquots of total lipid extracts were subjected to hydrogenolysis with $LiAlH_4$ (17). The resulting mixtures of long-chain alcohols and alkylglycerols were separated on layers of Silica Gel H with diethyl ether and identified by co-chromatography with standards. The fraction of alkylglycerols was treated with conc. HCl in order to remove plasmalogenic ether lipids from the alkylglycerol fraction by cleavage of the vinyl ether bond (18). The resulting reaction products, i.e., long-chain aldehydes, and the remaining 1-*O*-alkylglycerols were separated by TLC on Silica Gel H using hexane/diethyl ether (1:4, vol/vol), and identified by co-chromatography with standards. The fraction of 1-*O*-alkylglycerols was isolated and acetylated, and the resulting 1-*O*-alkyl-2,3-diacetylglycerols were analyzed by reverse phase (RP) HPLC as described in an upcoming section.

GC of fatty acid methyl esters and steryl acetates. GC of fatty acid methyl esters and steryl acetates was carried out using a Hewlett-Packard (Böblingen, Germany) HP-5890 Series II gas chromatograph. Fatty acid methyl esters were separated on a 0.24 µm Silar 5 CP WCOT fused silica column (Chrompack, Middelburg, The Netherlands), 50 m × 0.25 mm i.d., and steryl acetates on a 0.29 µm OV-1 fused silica column (Macherey & Nagel, Düren, Germany), 25 m × 0.32 mm i.d. Fatty acid methyl esters were separated using nitrogen (1.13 mL/min) as carrier gas initially at 165°C for 1 min, followed by linear programming from 165 to 205° at 1°C per min. The split ratio was 1:100, the injector temperature 230°C and the flame-ionization detector temperature 260°C. GC peaks were assigned by comparison of their retention times with those of known standards and by calculating equivalent chain lengths and relative retention times (relative to 16:0 = 1.00). Steryl acetates were separated under similar conditions using a linear temperature program from 228 to 252°C at 4°C per min. The final temperature was kept constant for 30 min.

GC/MS of steryl acetates. GC/MS of the steryl acetates was performed using the electron ionization mode (70 eV) and the chemical ionization mode (methane as reagent gas) on a Hewlett-Packard instrument Model 5890 Series II/5989 A. The GC instrument was equipped with a Permabond OV-1 fused silica capillary column (25 m × 0.32 mm i.d.; 0.23 µm film thickness). The carrier gas was He at a flow rate of 1.5 mL/min. The column temperature was initially kept at 225°C for 5 min and then programmed from 225 to 255°C at 4°C/min. The final temperature was held for 45 min. Other operating conditions were split/splitless injector in splitless mode (temperature 300°C), interface temperature 280°C and ion source temperature 200°C.

RP-HPLC of 1-O-alkyl-2,3-diacetylglycerols. A Merck-Hitachi (E. Merck) L-6200 pump equipped with an ACS 750/4 mass detector was used in combination with an integrator for monitoring HPLC separations and analysis of peak areas. 1-*O*-Alkyl-2,3-diacetylglycerols derived from ether glycerolipids were analyzed by RP-HPLC on

two LiChrospher 100 RP-18 (5 μm) columns (E. Merck), 125 × 4 mm each, in sequence using acetonitrile at a flow rate of 1.0 mL/min. Detector signals of the mass detector were quantified by calibration with synthetic standards.

RESULTS AND DISCUSSION

We have determined the total lipids of *P. ambiguus* (28 mg/g wet weight) and infected eel intestine (77 mg/g wet weight), as well as the triacylglycerols, which are the major neutral lipids of *P. ambiguus* (60 mg/g total lipids) and eel intestine (270 mg/g total lipids). In both organisms all other neutral lipid classes studied, such as wax esters, free fatty acids, sterols and steryl esters, were present only in minor proportions (Table 1). The fatty acid compositions of total lipids and various neutral lipid fractions, as well as the sterol compositions of free sterol and steryl ester fractions of *P. ambiguus*, were determined and compared with the corresponding lipid fractions of infected intestinal tissue of its host *A. anguilla*. The results we obtained on phospholipids will be reported in a subsequent paper (Aitzetmüller, Taraschewski, Filipponi, Werner and Weber, *Lipids*, to be published).

Sterols and steryl esters. Gas chromatographic analysis revealed that the major free and esterified sterol from both *P. ambiguus* and eel intestine is cholesterol. However, *P. ambiguus* has a much higher content of minor sterols than eel intestine and, consequently, a lower proportion of cholesterol (Table 2). Both intermediates of sterol metabolism and/or dietary phytosterols are found in the minor sterol fraction of the parasite, as was shown by GC/MS of their acetates. These minor steryl acetates show prominent $M^+ - 60$ (acetic acid) fragments that indicate the presence of the Δ^5-sterol structure. Molecular ions of 3-acetoxy derivatives are usually not detected in the mass spectra of Δ^5-unsaturated sterols because of the preferential formation of $\Delta^{3,5}$-diene derivatives by the loss of acetic acid (19). The most important mass spectral data are presented in Table 3.

The two compounds with retention times shorter than cholesterol have nearly identical mass spectra (Table 3). Beside the Δ^5 double bond of the sterol backbone, a second double bond must be present in the side-chain; this is evident from the fragment *m/z* 255, which is characteristic for a monounsaturated steryl backbone. It is known from the investigations of Itoh *et al.* (20) that only one pair of geometrical isomers of C_{27}-sterols exists with relative retention times (RRT) shorter than that of cholesterol (RRT = 1.00), i.e., Δ^{22}-dehydrocholesterols with a double bond having *E* or *Z* configuration at C-22 of the side-chain. Therefore, our results and the data given in the literature suggest the presence of both cholesta-5,22(*Z*)-dienol and cholesta-5,22(*E*)-dienol as constituents of *P. ambiguus* sterols.

The sterol with RRT 1.05 was identified as 7-dehydrocholesterol (Table 3), which has been frequently detected in the sterol fractions of various nematodes, e.g., *Caenorhabditis elegans* and *Turbatrix aceti* (21). The compound with RRT 1.07 was identified as a Δ^5-C_{28}-sterol with a further double bond in the side chain. These data agree, for example, with a dehydrocampesterol having one double bond in the side chain, whereas the sterol with RRT 1.16 was characterized as having a Δ^5-C_{28}-steryl moiety with a saturated side chain. One may speculate that this compound is a dehydrocampesterol with an additional double bond in the sterol nucleus. The mass spectrum of the compound with RRT 1.18 corresponds to that of a reference standard of campesteryl acetate (Table 3).

It is generally believed that acanthocephalans (1) and various other endoparasites (11) are not able to synthesize sterols *de novo*, particularly cholesterol. Obviously, cholesterol, which is the major sterol found in Acanthocephala, is absorbed from host intestinal contents together with other sterols, such as phytosterols. For example, Barrett *et al.* (22) have shown that cholesterol accounts for 57.5% of total sterols in *M. hirudinaceus* and 84% in *M. moniliformis*. In both parasites it is accompanied by appreciable proportions of campesterol (1–16%) and sitosterol (10–21%), as well as by trace amounts of stanols (dihydro sterols). The occurrence of various other sterols has been reported from a number of plant parasitic and free-living nematodes, e.g., *C. elegans* (21,23). These worms are able to metabolize exogenous sterols, e.g., by the introduction and isomerization of double bonds. The various sterols detected in the lipid extract of *P. ambiguus* most probably result from both nutrient uptake and metabolic changes. In contrast, the lipids of higher animals, such as eel, contain almost ex-

TABLE 1

Composition of Neutral Lipid Classes of Total Lipid Extract of *Anguilla anguilla* and *Paratenuisentis ambiguus*

Organism (tissue)	Neutral lipid classes				
	Sterols	Steryl esters	Wax esters	Triglycerides	Free fatty acids
	(mg/g total lipids)				
A. anguilla (infected intestine)	24	12	4	270	18
P. ambiguus (whole organism)	41	7	4	60	8

TABLE 2

Composition of Sterols and Steryl Esters of *Paratenuisentis ambiguus* and of Its Host *Anguilla anguilla*

Organism/sterol fraction	Composition (%) of sterols		
	Cholesterol	Campesterol	Other sterols
A. anguilla[a]			
Sterols	93.0	3.7	3.3
Steryl esters	94.6	2.0	3.4
P. ambiguus[b]			
Sterols	71.9	8.5	19.6
Steryl esters	80.1	4.4	15.5

[a]Infected intestine. [b]Whole organism.

TABLE 3

Mass Spectral Data of Steryl Acetates Derived from Sterols of *Paratenuisentis ambiguus*

Steryl acetate (RRT)[a]	Important mass fragments (m/z)[b]			Number of double bonds in nucleus	Number of double bonds in side chain
	$[M - CH_3COOH]^+$	$[M - CH_3COOH–CH_3]^+$	$[M - CH_3COOH$-side chain$]^+$		
Cholesta-5,22(*Z*)-dienol (0.90)	366 (100)	351 (19)	255 (46)	1	1
Cholesta-5,22(*E*)-dienol (0.93)	366 (100)	351 (15)	255 (39)	1	1
Cholesterol (1.00)	368 (100)	353 (19)	255 (14)	1	—
7-Dehydrocholesterol (1.05)	366 (97)	351 (26)	253 (53)	2	—
Δ^5-C_{28}-Sterol (with unsaturated side chain) (1.07)	380 (97)	365 (11)	255 (47)	1	1
Dehydrocampesterol (1.16)	380 (81)	365 (12)	253 (28)	2	—
Campesterol (1.18)	382 (100)	367 (18)	255 (15)	1	—

[a]Relative to cholesterol; RRT, relative retention time. [b]Intensities relative to base peak.

clusively cholesterol as the sterol component (>93–95%; Table 2).

Acyl lipids and free fatty acids. From studying the total lipids (Table 4), it was not clear whether the differences observed were simply due to different ratios of neutral lipids to phospholipids in parasite and host. For example, the data presented here show that the proportion of neutral lipids, in particular triacylglycerols, is higher by far in eel intestine than in the parasite. The results of fatty acid analyses of total lipids, wax esters, steryl esters, triacylglycerols and free fatty acids are summarized in Table 4. A number of GC peaks were believed to be minor fatty acids, including double bond positional isomers, and were not further identified (they are not included in Table 4). The figures represent area% (uncorrected) of the fatty acid methyl ester peaks.

There is a striking difference between the acyl moieties of steryl esters of eel gut, which contained predominantly 16:0 and 18:0 saturated fatty acids, and the acyl moieties of steryl esters of *P. ambiguus*, in which saturated fatty acids are present in minor proportions only. Major unsaturated fatty acids detected in the steryl ester fraction of *P. ambiguus* are 18:1n-9, 18:1n-7, 18:2n-6 and 20:5n-3 (Table 4).

The fatty acid compositions of wax esters isolated from *P. ambiguus* and eel intestine are also different in many respects (Table 4). Monounsaturated n-7 fatty acids (16:1n-7, 18:1n-7) are prominent acyl moieties in the parasite wax esters, but are present in low proportions only in wax esters of eel intestine. In *P. ambiguus* wax esters, part of which may originate from the parasite's surface coat, we found 20:1n-7 and 20:1n-11 acyl moieties that are not observed in the corresponding eel lipids. The same is true for 20:2n-6. The long-chain saturated fatty acids 20:0 and 24:0 are present in wax esters and steryl esters of both species.

Palmitoleic acid (16:1n-7) is a major fatty acid in all the *P. ambiguus* lipid classes and is present in amounts similar to those of 16:0. The ratio of peak areas of 16:0 to 16:1n-7 was fairly constant and close to 1 in all the parasite neutral lipid classes. This ratio was approximately 27 in the eel wax esters and 5.6 in the eel steryl esters. However, in the eel triglycerides, 16:0 and 16:1n-7 were again present at similar levels (11–12% of total fatty acids each). The critical pair 20:5n-3/22:0 (not separated on the Silar GC column) was estimated to be predominantly 20:5n-3, with the exception of *P. ambiguus* wax esters, where 20:5n-3 and 22:0 were present at a ratio of 2:1.

Among acanthocephalans, steryl esters and wax esters have been detected in *Polymorphus minutus* (24) and may be ubiquitously distributed in fish (25,26). However, little is known on the fatty acid composition of these lipid classes in acanthocephalans, except that the wax esters of *P. minutus* cystacanths, which reportedly occur in extremely high proportions, may be used as storage lipids by the larvae of this parasite (24).

The triacylglycerols of both parasite and host also show a complex pattern of acyl moieties. Although the two species contain triacylglycerols with remarkably high proportions of long-chain acyl moieties, it is obvious that peaks representing unsaturated fatty acids of chain lengths 16, 18 and 20 are more prominent in triacylglycerols of *P. ambiguus* than of eel intestine. Similar results have been found for the neutral lipid fractions of *M. moniliformis* (Acanthocephala) and *Oochoristica agamae* (Cestoda) (27,28).

To our knowledge, this is the first report on the fatty acid composition of triacylglycerols in adult acanthocephalans, whereas more information on the composition of triacylglycerols of various fish species, including eel, is available (4,6,29). It is evident from the studies on

TABLE 4

Fatty Acid Composition of Total Lipids and Various Neutral Lipid Fractions of *Anguilla anguilla* and *Paratenuisentis ambiguus*[a]

Fatty acid	Fatty acid composition (%) in									
			Neutral lipids							
	Total lipids		Steryl esters		Wax esters		Triglycerides		Free fatty acids	
	A	P	A	P	A	P	A	P	A	P
12:0	0.1	—	0.6	—	0.4	—	0.2	0.1	0.1	trace[a]
14:0	3.8	2.6	1.6	1.0	2.0	1.0	4.9	5.2	3.8	4.2
14:1n-5[b]	0.1	—	1.4	0.1	0.3	0.3	0.2	0.1	0.5	0.6
15:0	0.5	0.2	0.7	0.1	0.9	0.4	0.5	0.4	0.5	0.2
15:1	trace	—	0.4	—	0.9	0.3	0.1	—	0.1	—
16:0	15.9	6.2	20.1	2.9	13.5	9.7	11.0	9.8	23.6	7.3
16:1n-9	0.7	0.4	0.9	0.5	2.5	1.2	0.8	0.6	0.7	0.4
16:1n-7	9.1	5.4	3.6	2.9	0.5	7.0	11.9	10.0	9.7	6.5
16:1n-5[b]	0.3	0.2	—	—	2.3	1.4	0.4	0.7[c]	0.5	0.6[c]
17:0[d]	2.3	1.4	0.8	0.9	0.6	1.6	3.1	2.1	2.0	1.5
16:3n-4[b]	1.3	0.6	—	—	—	—	2.0	1.0	0.4	0.6
17:1[e]	0.1	0.1	0.6	0.2	0.6	0.7	0.2	0.2	—	—
18:0	5.1	8.8	36.9	2.0	19.4	6.9	1.8	3.3	13.6	9.9
18:1n-9	23.0	9.1	1.2	15.8	6.8	11.5	18.2	17.7	18.1	10.1
18:1n-7	6.8	6.5	1.5	13.2	1.3	9.1	5.0	7.9	10.2	7.3
18:1n-5[b]	0.2	0.1	—	—	0.6	0.3	0.2	0.2	0.3	0.1
18:2n-6	3.6	5.1	0.4	18.7	5.7	5.5	4.7	7.1	4.1	5.4
19:0[f]	0.5	0.9	—	1.2	—	1.2	0.5	0.8	0.6	1.0
18:3n-6	0.2	0.2	—	—	0.5	—	0.3	0.2	0.1	0.2
18:3n-4[b]	1.2	0.6	0.6	0.5	0.9	0.5	1.9	1.0	0.4	0.6
18:3n-3	0.8	0.9	0.7	3.2	0.6	0.7	1.6	1.1	0.1	0.8
19:1[b]	0.1	0.3	—	—	—	—	0.2	0.2	—	—
18:4n-3	0.4	0.8	—	1.1	—	0.4	0.6	0.8	—	0.5
20:0	0.2	4.0	2.5	0.4	1.1	5.7	0.1	0.6	0.5	4.2
20:1n-11	0.6	1.3	—	0.4	—	3.2	0.3	1.5	0.2	1.4
20:1n-9	0.9	1.1	0.7	1.2	1.0	3.2	0.5	0.8	0.6	1.2
20:1n-7	0.3	1.0	—	0.5	—	2.6	0.1	0.4	0.2	1.1
20:2n-6	0.6	1.1	—	1.8	—	3.1	0.5	1.1	0.6	1.1
20:3n-6	0.4	0.4	—	0.4	—	0.5	0.5	0.4	0.4	0.4
20:4n-6	2.2	4.9	6.0[c,h]	3.2	4.8[c,h]	1.3	2.0	2.5	1.7	4.4
20:3n-3	0.2	0.2	—	—	—	—	0.2	0.2	—	0.2
20:4n-3	0.7	0.4	—	0.4	—	0.4	1.2	0.5	—	0.3
20:5n-3[g]	4.8	23.5	0.6	22.0	0.4	3.1	7.1	11.1	0.7	17.5
22:4n-6	0.4	0.4	—	—	—	trace	0.4	0.3	0.2	0.3[c]
24:0	trace	0.8	2.8	0.9	5.4	2.8	0.3	0.3	0.2[c]	0.6
22:5n-3	2.3	1.7	—	0.4	—	0.8	3.8	1.6	0.4	1.3
22:6n-3	2.7	2.6	0.9	1.0	0.9	2.1	3.5	1.9	0.3	1.9
Others	7.7	6.2	14.5	3.1	26.1	11.5	9.2	6.4	4.4	6.3

[a]Abbreviations: A, *A. anguilla* (infected intestine); P, *P. ambiguus* (whole organism). Trace, <0.1%.

[b]Tentative identification.

[c]Partial overlap with a second peak or shoulder.

[d]May include 16:2n-4.

[e]May include 16:3n-3.

[f]May include 18:2n-4.

[g]May include 22:0.

[h]Equivalent chain length calculations indicate that the major part of this gas chromatographic peak could be another fatty acid.

fish lipids that the fatty acid composition of triacylglycerols and other lipids is significantly affected by dietary lipids as well as environmental conditions, in particular, temperature (5,6,30).

In the free fatty acid fraction, as in wax esters, the proportions of fatty acids in the 18:4 to 20:2 range are clearly increased in *P. ambiguus* as compared to eel intestine (Table 4). The fatty acid composition of the *P. ambiguus* free fatty acid fraction apparently contains several fatty acids, e.g., 18:4n-3, 20:0, 20:1n-7 and 20:1n-11, which are not present or present only in much smaller amounts in host intestinal tissue. Eicosapentaenoic acid was found to be a major fatty acid of total lipids as well as of steryl esters, triacylglycerols and free fatty acids.

It might have originated from aquatic diet (5) and/or from bacteria of the intestine (31). Relatively high proportions of myristic acid were detected in the fractions of triacylglycerols and free fatty acids of both *P. ambiguus* and infected eel intestine. In both acanthocephalans and fish, little is known so far on the composition of free fatty acid fractions; although some information is available on the composition of free fatty acids of *P. minutus* cystacanths, which were found to contain large proportions of saturated long-chain fatty acids (24). In the endoparasitic acanthocephalans, it is also possible that the free fatty acid fraction, at least in part, may originate from fatty acids of eel intestinal contents that have been adsorbed onto the parasite's surface coat.

Ether lipids. Ether glycerolipids are ubiquitous in the animal kingdom (32–34). Although the contents of neutral ether lipids and ether phospholipids have been determined extensively in a great variety of fish (35), little is known about the occurrence of ether lipids in Acanthocephala (1,11). Beames and Fisher (2) identified small proportions of plasmalogens in both *M. hirudinaceus* and *M. moniliformis*. We have determined the composition of alkyl moieties of ether glycerolipids in total lipid extracts isolated from *P. ambiguus* and eel intestine. The most abundant alkyl moiety in *P. ambiguus* is 16:0 (38%), followed by 16:1 and 18:1 (25.5% each). In eel intestine, a similar distribution of these alkyl moieties has been detected (16:0, 37.7%; 16:1, 22.6%; 18:1, 37.7%). 1-*O*-Octadecyl moieties (18:0), which are found in remarkably high proportions (9.5%) in the parasite, are low in eel intestine (2%); smaller proportions (1.5%) of 1-*O*-eicosenyl moieties (20:1) are detected in *P. ambiguus* but not in eel intestine. Only traces of plasmalogens, if any, have been detected in the ether lipid fraction of *P. ambiguus*.

In conclusion, we have found that, compared to eel intestine, the sterol and steryl ester fractions of *P. ambiguus* are characterized by the occurrence of remarkably high proportions of phytosterols as well as by dehydro sterols. The neutral acyl lipid fractions of both *P. ambiguus* and eel intestine contain large proportions of unsaturated and, in particular, polyunsaturated fatty acids. The chain lengths of these polyunsaturated fatty acids are found to be predominantly $\geq C_{20}$. In comparison with eel intestine, the neutral lipids of the endoparasite contain higher proportions of polyunsaturated fatty acids. The principles that regulate the metabolism, as well as the absorption and distribution of individual fatty acids, in *P. ambiguus* are not known. It has been shown in aquatic animals that, besides membrane lipids, the fluidity of storage lipids, such as triacylglycerols and wax esters, was increased at lower temperatures due to accumulation of mono- and polyunsaturated long-chain moieties in these lipids (4). Similarly, differences in the composition of the 1-*O*-alkyl moieties of ether glycerolipids from *P. ambiguus* and eel intestine have been found; it is noteworthy that the ether lipids of the parasite contain higher proportions of the 1-*O*-octadecyl moiety.

Finally, it should be noted that neutral lipids, such as wax esters, steryl esters, triacylglycerols and free fatty acids, usually are components of the surface coat (36) that protects endoparasites from the host environment. Such lipids have been shown to be released by human blood flukes (*Schistosoma* ssp.) (37–39). Studies on the biophysical properties of these lipids show that they are able to diffuse within the plane of the surface of endoparasites (40). Surface lipids may also play an important role in the resistance of *P. ambiguus* to host cellular defense mechanisms which are characterized by an increase in leukocyte numbers in the infected intestinal wall (41,42).

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Identification of a Deoxyguanosine–Malondialdehyde Adduct in Rat and Human Urine

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In an ongoing study, rat and human urine have been examined for the presence of malondialdehyde (MDA) derivatives as indicators of the nature of lipid peroxidative damage caused by this compound *in vivo*. MDA in urine was found to be present mainly in the form of two lysine adducts, one acetylated and the other unacetylated, reflecting *in vivo* reactions with tissue proteins. Two minor metabolites were identified as adducts with the phospholipid bases serine and ethanolamine and a third one as an adduct with the nucleic acid base guanine. The identification of an MDA adduct with deoxyguanosine (dG–MDA) among the products of hydrolysis of rat liver DNA suggested the possible occurrence of this compound in urine. In the present study dG–MDA was identified in rat and in human urine, and a high-performance liquid chromatographic method utilizing fluorescence detection was developed for its estimation. The method is sensitive to 1 pmol of dG–MDA and requires a minimum of 1 mL of rat urine or 5 mL of human urine. Its rate of excretion by five-week-old rats (28.54 ± 2.28 nmol/kg/24 h) (mean ± SEM) was higher than that for nine-week-old rats (6.29 ± 1.02) and much higher than that for adult humans (0.40 ± 0.05). The results indicate that, as reported for 8-hydroxy-deoxyguanosine, dG–MDA excretion is related to metabolic rate. Excretion of dG–MDA by the rat, like the excretion of total MDA, declines during growth on a body weight basis at a rate similar to the decrease in resting energy metabolism. In contrast to other MDA derivatives excreted in rat urine, vitamin E deficiency had no effect on the excretion of dG–MDA. Together with evidence that the dG–MDA content of rat liver DNA likewise is unaffected by vitamin E depletion or by administration of catalysts of *in vivo* lipid peroxidation, these findings indicate that DNA is protected from lipid peroxidative damage, possibly through conservation of the vitamin E associated with the lipids of the nuclear membrane.

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In an ongoing study, the forms in which malondialdehyde (MDA) is excreted in rat and in human urine have been investigated as a source of information on the nature of its reactions with macromolecules *in vivo*. About 75% of the MDA in urine collected from rats fed an MDA-free diet was found to be present as two lysine adducts, one acetylated and the other unacetylated, reflecting *in vivo* reactions with tissue proteins (1). Three minor metabolites were identified as adducts with the phospholipid bases serine and ethanolamine, and the nucleic acid base guanine. Of the total MDA in rat urine, only 10–20% is normally present in the free form. Human urine contains similar metabolites, but the excretion of total MDA (free and acid hydrolyzable) is much smaller on a body weight basis, and little or no MDA (0–5%) is excreted in the free form. Over half of the lysine derivatives excreted in human urine originate in the diet, where MDA generated by the peroxidation of food lipids reacts with the lysine residues of food proteins from which it is released during protein digestion and absorbed as a lysine adduct (2). Unlike free MDA, the lysine adduct is not mutagenic.

The identification of guanine–MDA (Gua–MDA) in urine (3) and of an MDA adduct with deoxyguanosine (dG–MDA) in a hydrolysate of rat liver DNA (4) suggested the possible occurrence of this adduct in urine. In this paper, evidence is presented for the presence of dG–MDA in rat and human urine, and estimates are given for its rate of excretion.

EXPERIMENTAL PROCEDURES

Animal experiments. Twenty-four-hour urine samples were collected from five five-week-old rats (body weight about 100 g) that had been fed a purified diet containing 10% corn oil (5) supplemented with a high level of vitamin E (100 IU ppm dl-α-tocopheryl acetate) for two weeks postweaning. The samples were collected under toluene twice daily, filtered and stored at –20°C prior to analysis.

In a second experiment, a group of five weanling rats was fed the vitamin E-supplemented diet and another group was fed the same diet, except that vitamin E was deleted and natural corn oil was replaced with molecular-distilled corn oil (5). After six weeks, vitamin E depletion in the second group was confirmed using an autohemolysis test (6) (81% hemolysis vs. 17% in the controls). Both groups then were switched for 72 h to an MDA-free diet containing 10% hydrogenated coconut oil, and urine was collected for the final 48 h.

Twenty-four-hour urine samples were obtained from five healthy, middle-aged women consuming free-choice diets, and aliquots were frozen as described.

Urinalysis for dG–MDA. Three mL of rat urine was mixed with an equal volume of ethanol and stored overnight at 4°C. After centrifuging, a volume of supernate equivalent to 2 mL of urine was freeze-dried, and the residue was dissolved in 1 mL of water.

The sample was applied to a chromatographic system composed of an AG 1-X8 anion exchange membrane in the chloride form (Bio-Rad, Richmond, CA), an AG 50W-X8 cation exchange membrane in the sodium form (Bio-Rad)

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Abbreviations: dG, deoxyguanosine; dG–MDA, deoxyguanosine–malondialdehyde adduct; DIP–MS, direct input probe-mass spectrometry; G, guanosine; GC/MS, gas chromatograph/mass spectrometry; G–MDA, guanosine–malondialdehyde adduct; Gua, guanine; Gua–MDA, guanine–malondialdehyde adduct; HPLC, high-performance liquid chromatography; 8-OHdG, 8-hydroxy-deoxyguanosine; TBA, thiobarbituric acid; TMS, trimethylsilyl.

and a C_{18} μBondapak reverse-phase SEP-PAK® capsule (Waters, Millipore Corp., Milford, MA). These components were mounted, in tandem, on a 10 mL syringe. The system was developed with 2 mL water, and the anion exchange membrane was removed. The remaining components were washed with 7 mL saturated KCl solution, and the cation exchange membrane was removed. The reverse-phase capsule then was washed with 2 mL water followed by 3 mL methanol. The methanolic fraction, containing dG–MDA, was dried at 40°C under a stream of air, and the residue was dissolved in 500 μL of water.

Further purification was achieved using an isocratic high-performance liquid chromatography (HPLC) system (Waters) that included a reverse phase C_{18} μBondapak analytical steel column, a scanning fluorescence detector (Model 470) and an absorbance detector (Model 440). For fluorescence analysis, the excitation and emission wavelengths were set at 340 and 518 nm, respectively. Aliquots (100–200 μL) of the partially purified sample were injected onto the column, and the system was developed with 10% methanol in water at a flow rate of 1 mL per min. Major fluorescence peaks were observed during the first 10 min, followed by a 12–15 min peak that coincided with that of pure synthetic dG–MDA (R_t 10.9 min) and was collected for further characterization. Recovery of standard dG–MDA added to the sample. This fraction was collected, evaporated to dryness, and the residue was taken up in 500 μL of water. Aliquots (100–200 μL) were re-injected, and the column was developed with 15% methanol in water at a flow rate of 1 mL per min. A 7–10 min fraction was collected, dried, and the residue was dissolved in 500 μL water. Two fluorescent compounds present in the fraction were resolved by rechromatography on the same column using 30% methanol in water at a flow rate of 0.5 mL per min. One compound co-eluted with synthetic dG–MDA (R_t 10.9 min) and was collected for further characterization. Recovery of standard dG–MDA injected onto the column was essentially quantitative over the operational range, and overall recovery was 89%.

The amounts of dG–MDA found in human urine were markedly lower than those for rat urine, necessitating the use of a larger starting sample (10–15 mL). In addition, human urine samples varied unpredictably in their content of fluorescent compounds, probably of dietary origin, that interfered in the isolation and quantification of dG–MDA using HPLC with fluorescence detection. Absorbance detection gave a well-defined peak for dG–MDA, but was ten times less sensitive than fluorescence detection. In contrast to urine samples obtained from rats fed a standard diet, each sample of human urine presented a different analytical challenge.

Isolation of dG–MDA from urine. Rat urine (100 mL in two 50-mL aliquots) was chromatographed on a 2.3 × 45 cm, 200–400 mesh AG 1X8 anion exchange column as described previously (7). The column was developed using a linear salt gradient provided by 1 L of 0–1 M NaCl solution. Fractions 60–120 (5 mL each) were pooled and reduced to 10 mL *in vacuo* at 37°C. The concentrated eluate was applied to a C_{18} μBondapak reverse-phase glass column (2 × 10 cm, 55–105 μm) and washed with 50 mL water, followed by 50 mL of 10% methanol to remove residual salt. The column then was developed with 25% methanol in water, and 10-mL fractions were collected.

Fractions 5–7 were combined, evaporated to dryness *in vacuo* at 37°C, and the residue was dissolved in 2 mL chloroform/methanol/water (85:14:1, by vol). This mixture was used as the mobile phase for further purification of the sample on a Zorbax® silica gel glass column (1.2 × 14 cm, 55–105 μm). Fractions 6–10 (4 mL each) were pooled, dried as before, and the residue was taken up in 300 μL water. Ten 30-μL aliquots (each equivalent to 10 mL urine) were injected onto a reverse-phase C_{18} μBondapak column (3.9 × 300 mm, 10 μm) fitted with a Guard-Pak Holder® (Waters) charged with C_{18} inserts. The column was developed for 50 min using a linear gradient of 0–7.5% acetonitrile at a flow rate of 1.0 mL per min. Eluates were detected by absorption (254 nm) and fluorescence (340, 518 nm) detectors, and the isolation procedure was monitored throughout by co-chromatography of the eluates with pure synthetic standard. Based on its retention time (45 min), the 44–46 min fractions were collected.

Human urine (600 mL) was freeze-dried and the residue dissolved in 120 mL distilled water. After standing overnight in the cold, the sample was centrifuged for 15 min at 2,000 rpm, and 100 mL of the supernatant fraction, equivalent to 500 mL of urine, was used for the isolation of dG–MDA by the procedure described for rat urine.

Preparation of silyl derivatives. The fractions collected from the silica gel column were dried and derivatized in acetonitrile using *O-bis*(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane. The reaction mixture was dried *in vacuo,* and the residue was subjected to HPLC, gas chromatography/mass spectrometry (GC/MS) and direct input probe-mass spectrometry (DIP-MS) analysis.

RESULTS

The chromatograms obtained for a sample of synthetic dG–MDA, for the eluate equivalent to 20 μL of rat urine collected from the reverse-phase column and for the com-

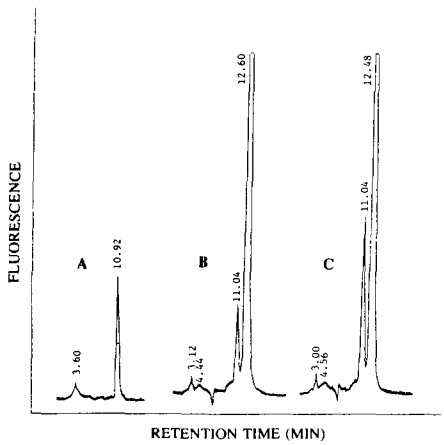

FIG. 1. High-performance liquid chromatography tracings obtained for 3.7 pmol of synthetic deoxyguanosine–malondialdehyde adduct (A), isolate equivalent to 20 μL of rat urine (B), and a combination of A and B (C).

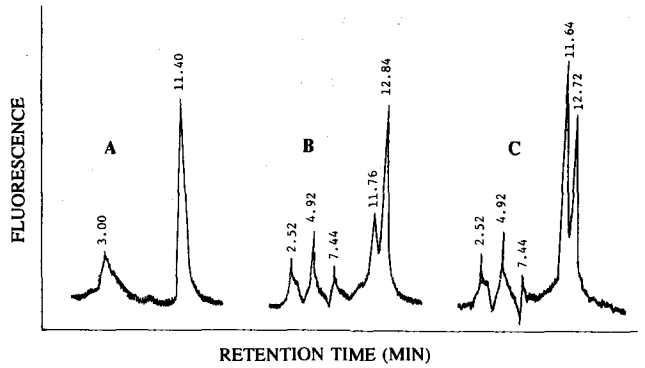

FIG. 2. High-performance liquid chromatography tracings obtained for 7.3 pmol of synthetic deoxyguanosine–malondialdehyde adduct (A), isolate equivalent to 100 μL of human urine (B), and the combination of A and B (C).

bined samples are illustrated in Figure 1. The analogous chromatograms for an eluate from human urine are shown in Figure 2. The fluorescence peak at *ca.* 12.5 min represented the only significant contaminant of dG–MDA found in the isolates from both rat and human urine and could not be consistently separated using reverse-phase HPLC. It did not appear in the absorption chromatograms, in which a single peak corresponding to that for synthetic dG–MDA was observed when a larger sample of urine was used.

The identity of the eluate from rat urine (Fig. 1B) was further investigated by examining its acid and thermal hydrolysis products. An aqueous solution of the compound was heated in a boiling water bath at pH 3 for 30 min in the presence of thiobarbituric acid (TBA), and the reaction products were analyzed for TBA–MDA complex by a procedure that is specific for MDA (8). The amount of isolate consumed in the reaction was quantified in terms of dG–MDA equivalents from a regression line for fluorescence peak area vs. pmoles of synthetic dG–MDA injected onto the column. The fluorescence response was linear over the range employed (1–10 pmol). The molar ratio of the amount of dG–MDA estimated by fluorescence analysis and found by MDA analysis was 1:0.98.

Another sample of isolate, estimated by fluorescence analysis to contain 44.16 pmol of dG–MDA, was partially hydrolyzed by heating in 100 μL of aqueous HCl in a boiling water bath for 30 min. The products were chromatographed on the C_{18} μBondapak column using 10% methanol in water as solvent. Absorbance peaks were recorded using a detector set at 254 nm and at maximum sensitivity. The amounts of Gua, deoxyguanosine (dG) and Gua–MDA in the hydrolysate were determined from standard curves prepared using pure compounds. The analysis yielded 23.76 pmol Gua, 5.26 pmol dG and 11.78 pmol of Gua–MDA (recovery 40.8 pmol or 92.4%).

Analysis of 24-h urine samples collected from five-week-old rats averaging 99 g in body weight yielded a value for dG–MDA of 28.54 ± 2.28 nmol/kg/24 h (mean ± SEM) (n = 5). The corresponding value for five 24-h samples of adult human urine was 0.40 ± 0.05 nmol/kg/24 h. The minimum operational sample size was 1 mL for rat urine and 5 mL for human urine.

Analysis of urine collected from five nine-week-old rats fed the vitamin E-deficient diet for the previous six weeks yielded a value of 6.29 ± 1.02 nmol dG–MDA/kg/24 h. The value for the positive controls was not significantly different (6.07 ± 0.69 nmol/kg/24 h). There was also no difference in final body weights (*ca.* 170 g). As found previously (5), the excretion of total MDA in the urine was higher in the deficient group (13.9 vs. 8.1 μg/24 h; $P < 0.05$).

The isolates from rat and human urine exhibited similar retention times when chromatographed on the reverse-phase column using a 0–7.5% acetonitrile gradient and on a Zorbax® silica column (4.6 × 250 mm, 10 μm) using a chloroform/methanol/water (93.6:6:0.4) mixture (*ca.* 44.5 and 20.4 min, respectively). Their trimethylsilyl (TMS) derivatives likewise had similar retention times (*ca.* 16.5 and 15.7 min, respectively) using a 2-propanol/methanol/*n*-hexane (20:5:75) mixture. These values coincided with those for synthetic dG–MDA and its TMS derivative, and a mixture of the isolates and their synthetic counterparts co-eluted.

The total ion chromatogram for the TMS derivative of synthetic dG–MDA (TMS–dG–MDA) subjected to GC/MS indicated that it was totally decomposed on the GC column. Ions were seen at *m/z* 187 (Gua–MDA) and at 73 (TMS). When analyzed by DIP-MS, peaks were seen at *m/z* 447 (molecular ion), 187 and 73. The spectrum for the synthetic compound was fully consistent with that found by Seto *et al.* (9). DIP-MS of the isolate from rat urine yielded ions at *m/z* 447 and 187. The presence of these ions is further evidence for the occurrence of dG–MDA in rat urine and for its pyrimidinopurine structure (9). The amount of isolate recovered from human urine (60 ng from 360 mL) was inadequate to obtain an interpretable spectrum.

DISCUSSION

The presence in urine of dG–MDA is consistent with its presence in an hydrolysate of rat liver DNA (4). The urinary compound is presumably a product of DNA repair synthesis. The excretion of dG–MDA by young, growing rats was 70-fold greater than that by adult humans on a body weight basis. Rodents excrete larger amounts of other products of oxidative damage, including thymine glycol and 8-hydroxydeoxyguanosine (8-OHdG) (10). The higher rate of excretion of these metabolites by rats is attributable to increased hydroxyl radical production associated with their high rate of oxygen consumption per unit body weight. As hydroxyl radicals are potent initiators of lipid peroxidation, their higher rate of dG–MDA excretion is attributable to the same cause.

There is other evidence for a higher rate of lipid peroxidation in rats than in humans. A greater proportion of the total MDA in rat urine is excreted in the free form (*ca.* 20 vs. 0–5%), and this fraction is markedly increased by the administration of iron nitrilotriacetate (5). The rate of excretion of dG–MDA found for human adults (404 ± 46 pmol/kg/24 h) is not markedly higher than that reported for 8-OHdG (~220 pmol/kg/24 h) (11). In contrast, the rate of excretion of dG–MDA by five-week-old rats was about 60-fold greater than that observed for 8-OHdG

excretion by two-month-old rats (11). This difference in the relative rates of dG–MDA and 8-OHdG excretion by the two species suggests that, at least in the rat, propagation by a free radical chain reaction, rather than initiation by hydroxyl radicals, is the main mechanism by which lipid peroxidation is driven. However, this explanation assumes that repair synthesis of DNA that has been modified by reactions with hydroxyl radicals and with MDA proceeds at the same rate.

Age is another probable factor in the difference in the dG–MDA content of rat and human urine found in this study. Total MDA excretion per unit body weight decreases progressively with age in rats, from 40 ng/g/24 h at three weeks of age to 25 ng at six weeks and 10 ng at 20–30 weeks (5). This decline is analogous to the decrease in the resting metabolism of growing rats from 260 kcal/kg/d at 40–42 days of age to 155 kcal at 60–64 d and 113 kcal at 100–120 d (12). The difference in dG–MDA excretion between the five-week-old and nine-week-old rats in the present study further indicates that during growth there is a steadily decreasing titer of free MDA in the tissues that is available for reactions with DNA Gua, the lysine residues of proteins and the amino groups of other compounds. Evidence for increased levels of lipid peroxidation products in the tissues of aged animals may be due to an increase in the production of free MDA production in old age or to an accumulation of these products during adult life.

The failure of vitamin E depletion to increase dG–MDA excretion in the rat is noteworthy in view of increases seen in the excretion of free MDA and its lysine derivatives in rats rendered vitamin E-deficient or treated with iron (5). However, it is consistent with a failure to find any change in the dG–MDA content of hydrolysates of DNA isolated from the liver of rats subjected to the same treatments (Draper, Agarwal and Wee, unpublished results). DNA appears to be better protected from peroxidative damage caused by MDA than are tissue proteins, possibly by conservation of vitamin E in the nuclear membrane. Vitamin E depletion of the rats used in this study was verified by liver analysis. The results of the urinalysis for dG–MDA indicate that the excretion of this compound is not a useful index of *in vivo* lipid peroxidation. The cause of significant disparities found in the dG–MDA content of DNA isolated from different rat organs and at different ages (Draper, and Wee, unpublished results) is unknown.

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COMMUNICATION

Fatty Acid and Positional Selectivities of Gastric Lipase from Premature Human Infants: *in vitro* Studies

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Gastric lipase activity in aspirates from premature human infants was tested for fatty acid and positional selectivity using racemic diacid triacylglycerols (TG) as substrates. The resulting free fatty acids and monoacylglycerols (MG) were recovered and analyzed. Octanoic acid (8:0) and decanoic acid (10:0) were hydrolyzed with a preference of 61.5:1 and 2.4:1 compared to palmitic acid (16:0) from *rac*-16:0-8:0-8:0 and *rac*-16:0-10:0-10:0, respectively. The ratio of lauric acid (12:0) to oleic acid (18:1) hydrolyzed from *rac*-18:1-12:0-12:0 was 13:1. Myristic acid (14:0), 18:1 and linoleic acid (18:2) were released at similar rates. These data and the composition of the MG suggest that, *in vitro*, the lipase is selective for shorter chain fatty acids and for fatty acids on the primary positions of the TG backbone.
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The lipase activity in human gastric aspirates was once believed to be of lingual origin. It was also thought that the enzymes that act in the stomach have little effect on dietary triacylglycerols (TG). In the human, the enzyme is now known to be secreted mostly from gastric mucosal cells (1,2). In the newborn, the enzyme hydrolyzes 40–60% of dietary TG (3,4). The lipid globules in milk from humans (4–6) and other species (4,7) are resistant to intestinal lipolysis by either pancreatic lipase (8–10) or bile salt-stimulated lipase from milk (5–7,11), unless first exposed to gastric lipases. Therefore, gastric lipase has a major role in the digestion of dietary TG, but it has received relatively little attention.

Specificity, now more commonly termed selectivity signifying a broader range, is one of the characteristics of lipases. Selectivity controls the digestion of TG and the absorption of the resulting free fatty acids (FFA) and monoacylglycerols (MG) (12). We found that the gastric lipase activity was stereoselective for the *sn*-3 ester over the *sn*-1 ester at a ratio of 4:1 using three enantiomeric TG containing 16:0 and 18:1 as substrates (13). During the present study, selectivity for shorter chain fatty acids was observed when we employed various synthetic TG as substrates. In this paper, we present data that show that, *in vitro*, human infant gastric lipase has a preference for the primary ester groups of TG and in varying degrees for 8:0 through 12:0 fatty acids over 16:0 and 18:1. The implications of our *in vitro* results and their relevance to *in vivo* lipolysis will be discussed. A preliminary report has been published (14).

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Abbreviations: FFA, free fatty acids; GLC, gas–liquid chromatography; MG, monoacylglycerols; *rac*-16:0-16:0-18:1, 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol; TG, triacylglycerols; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Aspirates containing gastric lipase were obtained as part of routine postnatal care at the Georgetown University Hospital (Washington, D.C.). The 0.5–5.0 mL samples were taken from infants whose gestational age was 33–42 wk. The specimens were obtained with the permission of the Georgetown University Institutional Review Board and the informed consent of the infant's parents or guardians. The samples were assayed for lipolytic activity (15), and the 32 samples with the highest activity levels were frozen on dry ice and sent to the University of Connecticut. All samples arrived frozen and were stored in a freezer at –70°C.

The digestion contained 0.1% citrate-Na_2HPO_4 buffer at pH 5.4, 5% bovine serum albumin and 0.1% gum arabic in a final volume of 25–50 mL. Prior to digestion, 2% of the desired TG was added, melted by heating on a steam bath if necessary, and emulsified with a Branson Sonifier (Branson Ultrasonics Corp., Danbury, CT). The mixture was equilibrated at 37°C in a water bath, and the gastric aspirate was added. The amount of aspirate used varied depending on the volume of the sample, but usually two equal portions were employed, one for the TG and one for a control without substrate. This procedure was necessary, as was the later addition of an internal standard, because each aspirate contained some lipid and the risk of denaturing the lipase by extraction of the aspirates with solvent was to be avoided. The incubation time was 30–60 min, depending on the activity of the aspirate. The racemic TG used as substrates were 16:0-8:0-8:0, 16:0-10:0-10:0, 18:1-12:0-12:0, 18:1-14:0-14:0, 18:1-18:2-18:2 and 18:2-18:1-18:1. The TG were synthesized as described by Jensen and Pitas (16). Purity of the TG was ascertained by thin-layer chromatography (TLC) and positional integrity was verified by pancreatic lipolysis (16).

After the desired period of incubation, i.e., upon about 40% hydrolysis, the samples were extracted, the digestion products were separated by TLC, and the fatty acids were analyzed by gas–liquid chromatography (GLC) after conversion to methyl esters (17). The products that contained 8:0 and 10:0 were converted to butyl esters. A known amount of methyl or butyl heptade-

canoate was added to each fraction prior to analysis by GLC. The fatty acids in the aspirate controls were subtracted from those in the respective digestion products after equalizing the internal standards and adjusting the amounts of the other acids accordingly. All data are given as mol%.

We did not attempt to separate the *sn*-1(3)-MG and 2-MG because, under our digestion conditions, acyl migration of the *sn*-2 ester to the *sn*-1(3) ester was observed.

RESULTS AND DISCUSSION

The fatty acid compositions of the original TG and of the MG and FFA from the digestions of the TG by gastric lipase are presented in Table 1. Included for validation of the TG structures are earlier data obtained by pancreatic lipolysis with the appropriate TG acids at *sn*-1(3), in FFA and in *sn*-2 MG. Selectivities for 8:0, 10:0 and 12:0 are clearly seen for gastric lipase. If there was no fatty acid selectivity, and with a 4:1 stereoselectivity for *sn*-3, the FFA from *rac*-16:0-8:0-8:0 would contain a 1:1 ratio of 16:0 and 8:0 (a 1:4 ratio for 16:0 and 8:0 from *sn*-16:0-8:0-8:0, and a 4:1 ratio for 16:0 and 8:0 from *sn*-8:0-8:0-16:0). The MG should be 100% 8:0. These theoretical ratios would apply to the digestion products from all the TG, but they did not for TG containing 8:0, 10:0 and 12:0 fatty acids. The ratios of fatty acids from these TG were 61.5:1 for 8:0/16:0, 2.3:1 for 10:0/16:0 and 13:1 for 12:0/18:1, all indicating selectivity for the shorter chain acids. Myristic acid (14:0) was not preferentially hydrolyzed compared to 16:0, nor was there selectivity between 18:1 and 18:2. The nature of the long-chain fatty acid seems to influence selectivity, e.g., 16:0-10:0-10:0 vs. 18:1-12:0-12:0.

The nature of the fatty acids in the MG (Table 1) suggests that apparent hydrolysis of the *sn*-2 ester occurred after acyl migration to *sn*-1(3). Our digestion conditions, pH 5.4 for 30 to 60 min, favor acyl migration; thus isolation and identification of the MG isomers would not have shed light on the mechanism. Nevertheless, some hydrolysis of 8:0, 10:0 and 12:0 at *sn*-2 probably occurred. We know from past experience with pancreatic lipolyses that acyl migration begins after about 5 min of digestion, which may also have occurred in the present case.

Lack of selectivity for the primary esters in TG of longer chain fatty acids is indicated by the approximately equimolar amounts of 18:1 and 14:0, and 18:1 and 18:2 in the FFA released from 18:1-14:0-14:0, 18:1-18:2-18:2 and 18:2-18:1-18:1. However, the MG again contained *sn*-1(3) fatty acids, which could be caused by either or both *sn*-2 hydrolysis and acyl migration. To differentiate between the two, we could have used triacid TG or, as has been recently described by Rogalska *et al.* (18), shorter digestion times that are less likely to produce MG, followed by high-performance liquid chromatography of the enantiomeric diacylglycerols derivatives. Rogalska *et al.* (18) did not observe any digestion of the *sn*-2 acid in 18:1-18:1-18:1 after 6 min of hydrolysis and confirmed the stereoselectivity of purified human gastric lipase for *sn*-3 of 18:1-18:1-18:1 by their method.

Our results are similar to those reported by other investigators (18,19). Thus, the rates of hydrolysis decreased as the chain length of the fatty acids in monoacid TG increased; the specific activities for 4:0-4:0-4:0 were 1000; for 8:0-8:0-8:0, 470; and for 18:1-18:1-18:1, 0.9 (18). The rates were dependent upon pH, the nature of the substrate and the type of enzyme, with a lower maximum, 4.5 vs. 5.5 for 4:0-4:0-4:0 than for 18:0-18:0-18:0 when homogenates of human gastric mucosa were used as gastric lipase source (19,20). With purified human gastric lipase, activity was only two times higher with 4:0-4:0-4:0 than with 18:1-18:1-18:1, and the pH optima were at 4.0 for Intralipid and at 4 to 6 for 4:0-4:0-4:0 (21). In this study, we did not compare the release of 16:0 and 18:1 from diacid TG containing these

TABLE 1

Fatty Acid Composition (mol %) of the Original Triacylglycerols (TG) and the Monoacylglycerols (MG) and Free Fatty Acids (FFA) Produced by Lipolysis with Premature Human Infant Gastric Lipase or Porcine Pancreatic Lipase

		Human gastric lipase[a]						Porcine pancreatic lipase[b]					
		TG		MG		FFA[c]		TG		MG		FFA[c]	
Substrates	n	X[d]	16:0 or 18:1	X[d]	16:0 or 18:1	X[d]	16:0 or 18:1	X[d]	16:0 or 18:1	X[d]	16:0 or 18:1	X[d]	16:0 or 18:1
rac-16:0-8:0-8:0	3	61.9	38.1	1.8	98.2	98.4	1.6	68.0	32.0	99+	—	53.6	46.4
rac-16:0-10:0-10:0	3	66.6	33.4	5.3	94.7	70.1	29.9	68.7	31.3	99+	—	54.0	46.0
rac-18:1-12:0-12:0	3	67.2	32.8	8.9	91.1	92.9	7.1	64.1	35.9	99+	—	52.5	47.5
rac-18:1-14:0-14:0	1	64.9	35.1	41.2	58.8	52.5	47.5	68.0	32.0	99+	—	53.4	46.4
rac-18:1-18:2-18:2	2	66.5	33.5	67.9	32.1	49.1	50.9	67.4	32.6	99.9	0.1	52.0	48.0
rac-18:2-18:1-18:1	2	33.0	67.0	70.9	29.1	43.3	56.7	34.2	65.8	99.6	0.4	49.2	50.8

[a]Lipase activity in gastric aspirates; lipolyses were done at pH 5.4 for 30 to 60 min to achieve about 40% hydrolysis.

[b]Porcine pancreatic lipase; lipolyses were done at pH 8.4 for 2 to 5 min.

[c]No fatty acid selectivity would be indicated by a 50:50 ratio, as seen for pancreatic lipase.

[d]X is the fatty acid in the TG, MG or FFA other than 16:0 or 18:1.

acids, but Hamosh *et al.* (22) observed preferential release of 18:1 and 18:2, as well as of 8:0 and 10:0 from infant formulas in the stomach. Further investigation of the apparent selectivity for unsaturated fatty acids is indicated. However, up to 60% of milk TG are hydrolyzed in the stomach of the suckling canine puppy, although canine milk fat contains almost exclusively longer chain fatty acids, none less than 14:0 (4).

Furthermore, there seems to be a temporal relationship to the type of fatty acid released: During short-term digestion (10 min) of human milk fat globules, the amount of 8:0 and 10:0 was higher than that of 18:1 and 18:2, whereas after 30 min of incubation, 18:1 and 18:2 far exceeded the level of medium-chain fatty acids released (23,24).

The extensive hydrolysis of milk fat in the stomach of several species whose milk contains exclusively or predominantly long-chain fatty acids, such as the dog (4), seal (24) and human (3), indicates that, *in vivo*, gastric lipase efficiently releases long-chain fatty acids from milk or formula (4,5,22,25,26). It is possible that the strong product inhibition of gastric lipase by long-chain fatty acids (19,27,28) does not occur under *in vivo* conditions (3,4). Furthermore, some dietary proteins have been shown to bind fatty acids more efficiently than does albumin, which is usually used as a fatty acid sink in *in vitro* studies (28). The presence of short-chain fatty acids at the *sn*-3 position of milk fat TG from the cow (29) explains their possible, but as yet undetermined, preferential release by gastric lipase, an enzyme with a 4:1 stereoselectivity for this position.

Our study, which shows that *in vitro* gastric lipase from premature human infants selectively releases 8:0, 10:0 and 12:0 as compared to 16:0 or 18:1 from racemic TG, emphasizes the need for careful *in vivo* examination of the selectivity of gastric lipolysis. The *in vitro* selectivity of the lipase can be summarized as follows: (i) positionally, primary esters are preferred; (ii) among fatty acids, short-chain fatty acids are preferred; and (iii) sterically, *sn*-3/*sn*-1 are preferred at a 4:1 ratio.

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Lymphatic Fatty Acids from Rats Fed Human Milk and Formula Containing Coconut Oil

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Human milk and infant formula containing coconut/soy oil were infused into the duodenum of rats to determine the incorporation of capric, lauric, myristic and palmitic acids into lymphatic triacylglycerol (TAG). The proportion of capric and lauric acids in the lymphatic TAG reflected the fatty acid composition of the diet. Based on positional analysis, it appears that more than 50% of the capric and lauric acids could have been absorbed from the intestine as *sn*-2 monoacylglycerols. In the rats fed human milk, 50% of palmitic acid in lymphatic TAG was in the *sn*-2 position. Because of the nonrandom distribution of palmitic acid in the lymphatic TAG, the nonspecific lipase in human milk, i.e., bile salt-stimulated lipase, did not appear to be a factor in milk lipid digestion.
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Human milk and infant formulas containing coconut oil have substantial amounts of capric (10:0), lauric (12:0), myristic (14:0) and palmitic acids (16:0). It is generally accepted that chain length determines the absorption pathway of these fatty acids with 10:0 and 12:0 being transported from the digestive tract in the portal blood while 14:0 and 16:0 are transported in the lymph (1,2). However, it is also known that the structure of the triacylglycerol (TAG) influences the absorption of component fatty acids (3–5). While most of the 10:0 and 12:0 from human milk and infant formula may be taken up into the portal blood, a significant portion may be transported in the lymph if absorbed from the intestinal lumen as *sn*-2 monoacylglycerol (MAG) and resynthesized into lymphatic TAG by the *sn*-2 MAG pathway.

Lipids from human milk contain 20–25% 16:0, which is located predominantly in the *sn*-2 position of the milk TAG (6). Due to the specificity of pancreatic lipase for hydrolysis of fatty acids from the primary positions of TAG, a large proportion of 16:0 from human milk may be absorbed as *sn*-2 MAG (4). However, human milk also contains bile salt-stimulated lipase (BSSL), a nonspecific lipase that is thought to complete digestion of milk lipid by hydrolyzing MAG (7). If BSSL contributes significantly to milk lipid digestion, 16:0 would be hydrolyzed from the *sn*-2 MAG produced by pancreatic lipase, and the final products of digestion would be primarily free fatty acids and glycerol (7). In the absence of *sn*-2 MAG, the phosphatidic acid pathway would be predominant in the enterocyte, and it is assumed that 16:0 would not be preferentially introduced in the *sn*-2 position of TAG by this pathway. Hence, there would be no enrichment of 16:0 in the *sn*-2 position of the lymphatic TAG in animals fed human milk, as was previously suggested (4).

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Abbreviations: BSSL, bile salt-stimulated lipase; MAG, monoacylglycerol; TAG, triacylglycerol.

In the present study, human milk and infant formula containing coconut/soy oil were infused into the duodenum of rats to determine incorporation of 10:0, 12:0, 14:0 and 16:0 into lymph and the position of these fatty acids in the lymphatic TAG. One objective of this study was to test the assumption that very little 10:0 or 12:0 is transported in the lymph. A second objective was to determine the position of 16:0 in the lymphatic TAG of rats receiving human milk. If enrichment of 16:0 in the *sn*-2 position is not maintained through digestion and absorption, it would indicate nonspecific lipase activity and support a role for BSSL in digestion of milk lipid. It is assumed that the phosphatidic acid pathway, which would become operative in the absence of 2-MAG, would not preferentially introduce 16:0 in the *sn*-2 position of the lymphatic TAG.

MATERIALS AND METHODS

Animals and surgical procedure. Male Holtzman albino rats obtained from Holtzman Laboratory Animals (Madison, WI) were used in this study. At the time of surgery the rats weighted 275–350 g, and they 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 Warsaw (8). First the rats were anesthetized with methoxyflurane, and a right subcostal incision was made. The major mesenteric lymph duct was located, and a bevelled heparin-filled micro-renathane implantation tube (MRE 040; Braintree Scientific, Braintree, MA) was threaded approximately 3 mm into the lymphatic duct. After a good lymph flow was established, the cannula was secured using cyanobutylacrylate adhesive. Then a feeding tube was placed in the duodenum by passing MRE 040 micro-renathane tubing in a caudal direction through a small gastric puncture and securing it with 4-0 silk. The abdominal wall was closed by suturing the muscle layer with 4-0 silk and then by stapling the skin with Michel wound clips (Harvard Bioscience, South Natick, MA).

Immediately following surgery, the rats were restrained in modified Bollman cages (9), placed in a warm dark environment and allowed to recover for approximately 24 h. During the recovery period the rats had access to water, and a glucose/electrolyte solution (Pedialyte; Ross Laboratories, Columbus, OH) was infused into the duodenum at 2.0 mL/h.

Infusates. Following their recovery, six rats were infused (2.5 mL/h) for 12 h through the feeding tube with human milk (3.3 g lipid/100 mL), and six rats were infused with an infant formula containing coconut/soy oil (3.4 g lipid/100 mL, ProSobee; Mead Johnson, Evansville, IN). Lymph was collected for 12 h in heparinized tubes on ice and stored at −70°C until analyzed.

The human milk was donated by a woman during her eighth month of lactation. Several complete breast ex-

pressions were collected with the aid of an electric breast pump. Milk samples were pooled and stored at –70°C.

Lipid analyses. Total lipids were extracted from aliquots of each diet and each lymph collection was made by a modified Folch procedure (10). The TAG in the lymph were isolated using Silica Gel G thin-layer chromatographic plates developed with hexane/diethyl ether/glacial acetic acid (90:30:2, by vol).

An aliquot of the TAG was used for structural analysis. The fatty acids esterified to the *sn*-2 position were measured directly after hydrolysis of the fatty acids from the *sn*-1,3 positions with porcine pancreatic lipase (11). The fatty acids esterified in the *sn*-1,3 positions were calculated as:

$$(sn\text{-}1,3) = \frac{3(\text{TAG}) - (sn\text{-}2)}{2} \quad [1]$$

Fatty acids associated with total lipid, TAG and *sn*-2 MAG were transmethylated in methanol/hexane (4:1, vol/vol) in the presence of acetyl chloride (12) 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).

RESULTS AND DISCUSSION

The fatty acid composition of the infused diets and of the lymph are shown in Table 1. The proportions of 10:0 and 12:0 in lymphatic TAG were approximately the same as those in the diets. This was unexpected as it was assumed that these fatty acids would be transported as albumin-bound free fatty acids in the portal blood (1,2).

One factor that discriminates against transport of 10:0 and 12:0 in the lymph is the preference of acyl-CoA synthetase for fatty acids containing more than 12 carbons (13). When dietary lipid is digested and the fatty acids are absorbed in free form, they must be activated by acyl-CoA synthetase before reesterification into lipid and subsequent transport in the lymph. In the enterocyte, the *sn*-2 monoglyceride pathway is the major pathway for TAG synthesis, and *sn*-2 MAG, a product of pancreatic lipase digestion, serves as the fatty acyl-CoA acceptor (14). Therefore, the fatty acids in the *sn*-2 position of the TAG synthesized by this pathway do not require activation by acyl-CoA synthetase, while fatty acids in the *sn*-1,3 positions do.

A positional analysis was made of lymphatic TAG to determine the distribution of fatty acids in the *sn*-2 and *sn*-1,3 positions (Fig. 1). In the human milk-infused animals, 83% of the 10:0 and 67% of the 12:0 were esterified to the *sn*-2 position of the lymphatic TAG. The TAG in the lymph of the formula-fed animals contained 50% of 10:0 and 54% of the 12:0 in the *sn*-2 position. It appears that a large proportion of the 10:0 and 12:0 from formula and human milk could have been taken up by the enterocyte as *sn*-2 MAG and resynthesized into lymphatic TAG. The 10:0 and 12:0 associated with the *sn*-1,3 positions would have required activation by acyl-CoA synthetase. While medium-chain fatty acids are not the preferred substrate for acyl-CoA synthetase, 10:0 and 12:0 can be activated, as suckling rats are reported to transport considerable 10:0 and 12:0 in the *sn*-1,3 positions of lymphatic TAG (15).

TABLE 1

Fatty Acid Composition of Infusate and Lymph (wt%)[a]

	Human milk		ProSobee[b]	
Fatty acid	Infused	Lymph	Infused	Lymph
10:0	1.0	0.4 ± 0.1	2.2	1.2 ± 0.5
12:0	6.2	3.4 ± 0.7	23.1	19.4 ± 1.4
14:0	7.2	5.8 ± 0.9	11.3	9.0 ± 0.5
16:0	19.9	24.3 ± 2.0	16.3	13.8 ± 1.8
16:1	1.6	2.6 ± 0.5	n.d.[c]	0.1 ± 0.1
18:0	9.4	9.2 ± 0.4	4.6	5.5 ± 0.3
18:1	31.9	29.9 ± 2.2	15.5	17.2 ± 0.8
18:2n-6	16.4	16.4 ± 1.5	25.8	28.4 ± 0.6
18:3n-3	1.1	0.8 ± 0.2	3.1	3.0 ± 0.4
20:4n-6	0.5	3.0 ± 0.5	n.d.	1.3 ± 0.4

[a]Lymph values are means of six observations ± standard deviation.

[b]ProSobee from Mead Johnson (Evansville, IN). The lipid formulation of this infant formula has changed since this study was undertaken.

[c]Fatty acid not detected (less than 0.05 wt%).

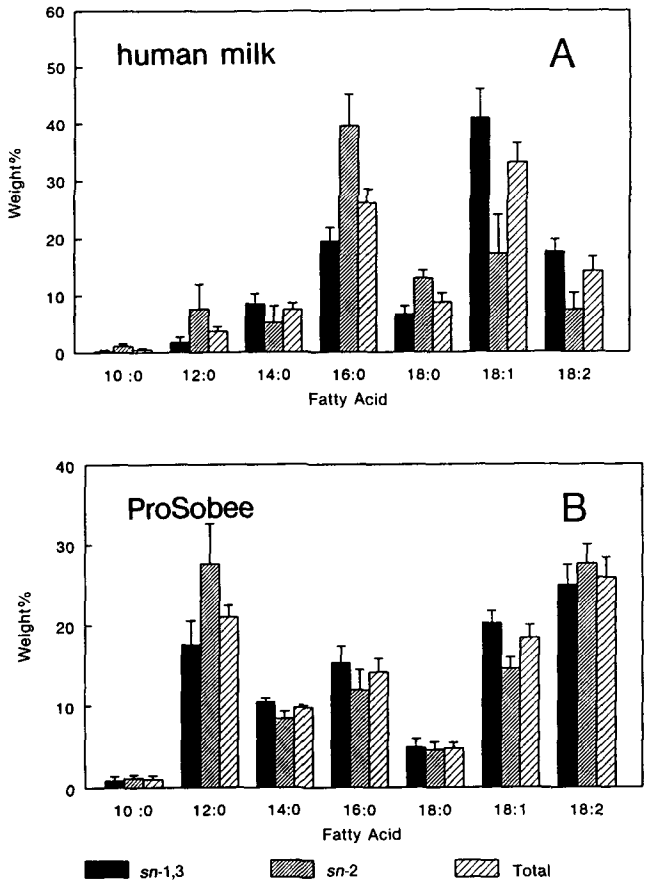

FIG. 1. Distribution of fatty acids (wt%) within each position of lymphatic triacylglycerol from six rats intraduodenally infused with (A) human milk or (B) infant formula (ProSobee; Mead Johnson, Evansville, IN). Results are presented as mean ± standard deviation, n = 6.

Unlike 10:0 and 12:0, the proportion of 14:0 was greater in the *sn*-1,3 positions. Myristic acid is a true long-chain fatty acid, and it is readily activated by acyl-CoA synthetase and incorporated into lymphatic TAG (13).

In the rats infused with human milk, the proportion of 16:0 in the *sn*-2 position of lymphatic TAG was much greater than seen in the rats infused with formula (Fig. 1). This was of interest because one proposed explanation for efficient absorption of milk lipid is the high proportion of 16:0 in the *sn*-2 position of human milk TAG (4). It is thought that 16:0 in the *sn*-2 position would be absorbed as *sn*-2 MAG and not be acted upon by pancreatic lipase and freed. As free 16:0 readily complexes with divalent cations, forming a soap that is poorly absorbed not being in free form would enhance its absorption.

Human milk contains an endogenous lipase, BSSL, i.e., a nonspecific lipase that releases fatty acids from all positions of TAG. It has been proposed that the improved absorption of human milk lipid is due to BSSL in the milk (7). If BSSL were a significant factor in the digestion of milk lipid, it would hydrolyze the product of pancreatic lipase, i.e., 16:0-enriched MAG, and subsequently decrease the proportion of 16:0 in the *sn*-2 position of lymphatic TAG. Because of the large amounts of 16:0 in the *sn*-2 position of lymphatic TAG, BSSL did not appear to be an important factor in the digestion of milk lipid. However, the conditions in this study are not the same as seen in the neonate. In this study, diets were infused into the duodenum so that lipolysis was not initiated by gastric/lingual lipase in the stomach. It has been suggested that circumventing gastric lipolysis in the infant may reduce lipid absorption by 30% (7). Also BSSL may only contribute to milk lipid digestion when bile salts in the intestine are below the critical micelle concentration, and pancreatic lipase is less than optimal (7). These are not conditions in the intestinal lumen of the adult rat. Because of these differences, the results of the present study do not negate a possible role for BSSL in the infant.

In conclusion, significant amounts of 10:0 and 12:0 from human milk and infant formula can be absorbed and transported from the gut *via* the lymph system. The amount of 10:0 and 12:0 found in the lymph is proportional to the amount in the diet with more than 50% esterified to the *sn*-2 position of the lymphatic TAG. The large proportion of 16:0 in the *sn*-2 position of the lymphatic TAG from animals infused with human milk does not support a role for BSSL in the digestion of milk lipid in the adult rat.

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Confirmation by Gas Chromatography/Mass Spectrometry of Two Unusual *trans*-3-Monoethylenic Fatty Acids from the Nova Scotian Seaweeds *Palmaria palmata* and *Chondrus crispus*

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The structures of two unusual fatty acids, the known *trans*-3-hexadecenoic acid and a novel *trans*-3-tetradecenoic acid, both isolated from the Nova Scotian seaweeds *Palmaria palmata* and *Chondrus crispus*, were positively identified. After the extraction of the total fatty acids by saponification, followed by methylation, the monoenoic *trans* fractions were isolated by thin-layer chromatography on silica gel impregnated with silver nitrate. The monoenoic *trans* fractions were derivatized with 2-amino-2-methyl-propanol prior to analysis by gas chromatography/mass spectrometry. The mass spectra showed prominent $[M + 1]^+$ ions but lacked the 12 amu interval useful for identifying the double bond position. Hence, alternative diagnostic peaks were used to confirm the position of the double bond in these two fatty acids. As the *trans*-3-hexadecenoic acid is found in the photosynthetic tissue of all plants, it may also be present in ruminant fats and, presumably, in human adipose tissue.

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For nearly 30 years (1), *trans*-3-hexadecenoic acid has been known to occur as a component of plant photosynthetic lipids (2,3). This *trans* acid has been found among the fatty acids of numerous North Atlantic macrophytes (4–8), sometimes exceeding 2% of total fatty acids. We have now identified *trans*-3-tetradecenoic acid in several of these algal samples. The rigorous identification of the structure of two homologous fatty acids of this type (14:1, 16:1) from seaweeds by modern gas chromatography/mass spectrometry (GC/MS) is reported here.

MATERIALS AND METHODS

Seaweeds. *Palmaria palmata* and *Chondrus crispus* grown in natural seawater and provided through the courtesy of the Institute for Marine Biosciences of the National Research Council of Canada (Halifax, Nova Scotia, Canada) were the source of the *trans* fatty acids. 2-Amino-2-methyl-propanol (AMP) reagent was obtained from Sigma Chemical Co. (St. Louis, MO). *trans*-3-Hexadecenoic acid for use as a standard was synthesized in our laboratory by a recently published method (9); its structure was confirmed by oxidative ozonolysis (10), MS upon derivatization and infrared (IR) spectroscopy of the free acid (band at 975 cm^{-1}).

Extraction. The total fatty acids from fresh seaweeds were isolated upon saponification by refluxing with a 0.5 N methanolic sodium hydroxide for 2 h (6). After removing the nonsaponifiable components by extraction with hexane, the fatty acids were recovered after acidification by re-extraction with hexane, and then converted to their methyl esters by heating with 8% BF_3 in methanol at 100°C for 60 min (11).

Silver nitrate thin-layer chromatography (TLC). The methyl esters were applied to silica gel TLC plates (Adsorbosil-5; Alltech Associates Inc., Applied Science Laboratories, Deerfield, IL) impregnated with silver nitrate (10% in acetonitrile) (12) and fractionated according to their degree of unsaturation (13) by development in benzene/hexane (2:1, vol/vol). The monoenoic *trans* band (R_f = 0.87) was visible under ultraviolet (UV) light after spraying with 2′,7′-dichlorofluorescein (0.2% in ethanol). The adsorbent band was scraped off, and the silica gel was extracted three times with hexane/chloroform (1:1, vol/vol). The pooled extracts were evaporated to dryness under N_2 and redissolved in hexane for further analysis.

Structural identification as 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives. The DMOX derivatives of the fatty acids were prepared by adding 250 μL of AMP to 500 μg of the fatty acid methyl esters in screw-cap vials. After flushing with N_2, the vials were capped and heated at 180°C for 18 h (14). The reaction mixture was cooled, dissolved in 3 mL of dichloromethane and then washed twice with 1 mL of water. After drying the organic phase with anhydrous Na_2SO_4, the solvent was removed under a stream of N_2. The samples were dissolved in hexane prior to analysis by GC/MS.

GC/MS. Electron impact mass spectra were obtained on a model 700 Finnigan MAT Ion Trap Detector (ITD) system (Finnigan MAT, San Jose, CA) interfaced to a Perkin Elmer model 990 gas chromatograph. The gas–liquid chromatography (GLC) fused silica capillary column was fed through a heated transfer line directly into the ITD gas inlet. GC/MS separations were obtained on a flexible-fused silica nonpolar column (DB-1; coating of methyl silicone, 60 m × 0.25 mm i.d., phase thickness 0.25 μ; Chromatographic Specialties Inc., Brockville, Ontario, Canada). The column was operated at 220°C and at 140 kPa helium. The data system consisted of an IBM clone (dtk TECK 1230) interfaced to the internal electronics of the ITD using version 4.0 of the ITD software supplied by the Finnigan Corp. using background subtraction. The ITD was tuned by following the manufacturer's procedure and was operated in the full-scan mode with a 1-s cycle time.

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Abbreviations: AMP, 2-amino-2 methyl-propanol; DMOX, 4,4-dimethyloxazolines; ECL, equivalent chain length; GC/MS, gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; ITD, ion trap detector; TLC, thin-layer chromatography.

RESULTS AND DISCUSSION

In seaweeds, in which water is by far the major (90–95%) component, total fatty acids are present only in small amounts. In *C. crispus,* the total fatty acids amounted to 0.41% of the wet weight, and in *P. palmata* came to only 0.34%. *trans*-3-Tetradecenoic acid and *trans*-3-hexadecenoic acid are only minor components of the total fatty acids. The proportion of *trans*-3-tetradecenoic was 0.1% of total fatty acids in both algae, and the proportions of *trans*-3-hexadecenoic acid were 2.4 and 0.9% in *P. palmata* and *C. crispus* acids, respectively. These percentages and those of the other fatty acids were similar to those published earlier and differed only in that *C. crispus* had ~25% 20:4n-6 vs. 1% of this acid in *P. palmata.* Conversely, *C. crispus* had ~25% 20:5n-3, whereas *P. palmata* had ~45%. Neither contained 22:6n-3, and all other fatty acids were ≤6%, except for 16:0, which occurred at about 25% of total fatty acids in both macrophytes.

The mass spectra of the DMOX derivatives obtained with the ITD clearly identified the structures of the *trans*-3-tetradecenoic acid and of the *trans*-3-hexadecenoic acid. A prominent molecular ion and fragments characteristic of fatty acids with the double bond in position 3 were observed (Figs. 1 and 2). It should be emphasized here that a characteristic feature of the ITD is that instead of the molecular ion the protonated ion $[M + H]^+$ occurs (15).

trans-*3-Tetradecenoic acid.* The mass spectrum of the DMOX derivative of *trans*-3-tetradecenoic acid (Fig. 1) showed the protonated molecular ion at *m/z* 280, corresponding to a 14:1 fatty acid derivative with 279 atomic mass units (amu). The base peak was an ion at *m/z* 110, the composition of which is not well known (it appears to be derived from a parent ion fragment at *m/z* 152). This ion, after double bond isomerization and cyclization, produces a configuration that loses a $C(CH_3)_2$ group from the heterocyclic ring with loss of 42 amu (Fig. 3). This ion is typical (16) of the DMOX derivative of a fatty acid with a double bond in position 3, whether *cis* or *trans,* and it is not observed when the double bond is in position 2 or 4.

Another important ion, which appears at *m/z* 264, is due to the loss of a methyl group from the ring, whereas the ion at *m/z* 152 is due to a typical cleavage between the carbons in positions 5 and 6. The ion at *m/z* 113, which is a characteristic of DMOX derivatives of fatty acids, is not very abundant when the double bond is close to the polar end of the molecule. It is usually due to a McLafferty rearrangement and is formed by the migration of the γ-hydrogen followed by cleavage between the carbon atoms 2 and 3.

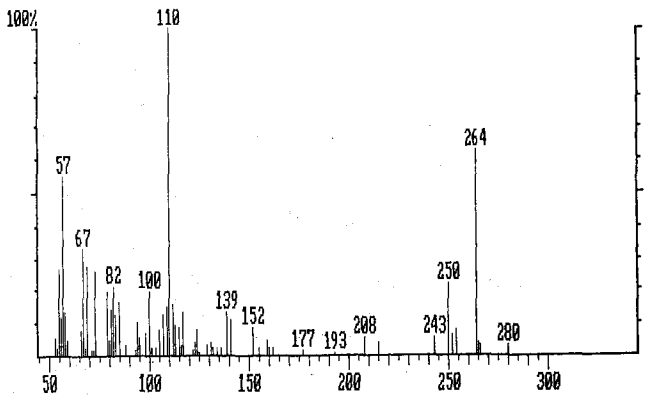

FIG. 1. Electron impact mass spectrum of the 4,4-dimethyloxazoline derivative of natural *trans*-3-tetradecenoic acid.

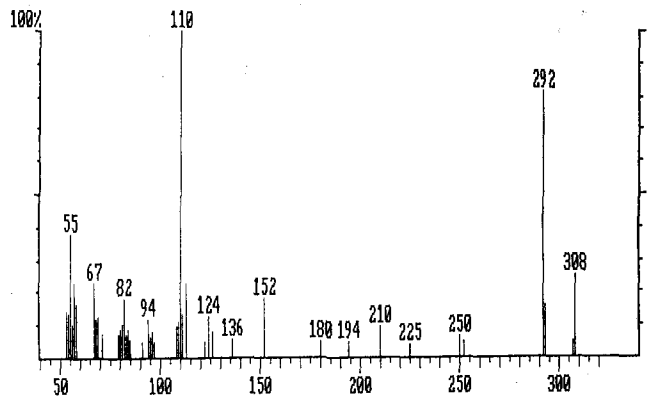

FIG. 2. Electron impact mass spectrum of the 4,4-dimethyloxazoline derivative of natural *trans*-3-hexadecenoic acid.

trans-*3-Hexadecenoic acid.* The electron impact mass spectrum of the DMOX derivative of the *trans*-3-hexadecenoic acid (Fig. 2) was very similar to the spectrum of the *trans*-3-tetradecenoic acid; the protonated molecular ion at *m/z* 308 was as expected. The base peak was at *m/z* 110 whose origin is presumably the same as for the other DMOX derivative discussed previously. A prominent ion at *m/z* 292 due to loss of a methyl group from the ring was also observed. Other important ions include that at *m/z* 152, resulting from a cleavage between carbon atoms 5 and 6, and the ion at *m/z* 113. The ion at *m/z* 126 is a cyclic ion formed by cleavage between carbons 4 and 5 (17).

The mass spectrum of the DMOX derivative of the reference standard *trans*-3-hexadecenoic acid (not shown) had the typical fragmentation pattern observed for the *trans*-3 fatty acids (DMOX derivatives) isolated from the seaweeds.

The mass spectra of the two *trans*-3 fatty acids investigated here permitted investigators to determine their structures, even though the rule of Andersson and Holman (18–20) for identifying ethylenic bond positions by gaps of 12 amu was not applicable. This rule generally applies to the DMOX derivatives of fatty acids with one or more ethylenic bonds but is not applicable to *trans*-3 fatty acids be-

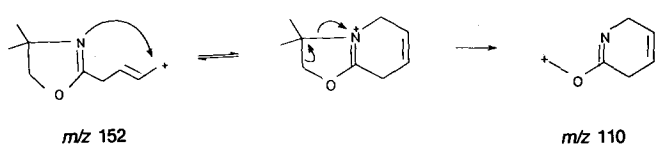

FIG. 3. Proposed mechanism for the formation of ion at *m/z* 110.

cause of the position of the double bond close to the polar end of the molecule. The spectra obtained with the ITD showed, however, a base peak at m/z 110, which is typical of these compounds with a double bond in position 3.

There are six fatty acids that commonly occur (21) in green vegetables, including *trans*-3-hexadecenoic acid. Few vegetables contain much 14:0, whereas the total fatty acids of the two seaweeds contain 4 *(C. crispus)* and 9% *(P. palmata)*. Both contain approximately 25% of 16:0. The *trans*-3 monoenoic contents of the total fatty acids given above do not correlate with the proportions of the corresponding saturated acids. The *trans*-3-tetradecenoic acid is not specific to the red seaweeds, but is also found in brown and green seaweeds.

The equivalent chain length (ECL) of the methyl ester of *trans*-3-hexadecenoic acid for two polyglycol-type liquid phases (SUPELCOWAX-10, Omegawax; Supelco Canada, Oakville, Ontario, Canada) was 16.51 at 185°C. The corresponding ECL value for the ester of *trans*-3-tetradecenoic acid was 14.51. The ECL values for these esters on the methylsilicone liquid phase (DB-1) at 200°C are, respectively, 13.90 (14:1) and 15.91 (16:1). The unique behavior of *trans* acids on cyanosilicone liquid phases (22), eluting earlier than the corresponding *cis* acids, will cause the ECL values to vary with cyano group content (23). One set of ECL values (7) for seaweed 16:1 fatty acid methyl esters separated on a 50 m × 0.25 mm stainless-steel open-tubular column with the cyanosilicone liquid phase SILAR-5CP illustrates this point: *cis*-9-hexadecenoic, 16.38; *trans*-3-hexadecenoic, 16.45; *cis*-13-hexadecenoic, 16.63. On the polyglycol columns, the tentative identification of *trans*-3-hexadecenoic acid in butterfat (24) placed it in the *iso*-17:0 position, approximately at ECL 16.46.

Interestingly, the deposition of *trans*-3-hexadecenoic acid in ruminant fat was forecast (2) as long ago as in 1964, but does not seem to have been addressed in human depot fats. It could be important, in view of current health recommendations to consume more green vegetables, to accurately identify this particular 16:1 fatty acid in total *trans* 16:1 of human adipose tissue. Total *trans* 16:1 levels (by packed column) are purported to be correlated with deaths from coronary heart disease (25,26).

The consumption of seaweeds as human food is common in many countries, especially in Asia (27,28), but there does not seem to be a record of the deposition of *trans*-3-hexadecenoic acid in the depot fats of such populations. Conversely, studies on partially hydrogenated edible oils (22,29) suggest that all *trans*-3 fatty acid isomers, as a result of the mobility of this bond in the proximity of the carboxyl group, would be unlikely to survive hydrogenation to any extent.

In humans, the 16:1 fatty acids of depot fats seem to be in the range of 2–6% of total fatty acids (30,31). These seem to be primarily derived from animal fats. In fish lipids, palmitoleic (*cis*-9-hexadecenoic) acid invariably constitutes an important proportion of the fatty acids (32). As edible vegetable oils are usually low in 16:1, these may be discounted as a source for *trans*-3-hexadecenoic acid originating from partial hydrogenation except if chain shortening (33,34) of the artifactual *trans*-5-octadecenoic acid takes place *in vivo*. However, the latter fatty acid seems rarely present among the isomers formed in the hydrogenation process when applied to vegetable oils (22,35). A chromatogram of methyl esters of fatty acids of human adipose tissue that has been published (30) shows two large 16:1 peaks, allegedly with the smaller n-7 isomer eluting ahead of the n-9 isomer, followed by a cluster of smaller peaks, one of which may represent *trans*-3-hexadecenoate.

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LETTER TO THE EDITOR

α-Linolenic Acid in Human Adipose Tissue

Dear Sir:

In a paper on site-specific differences in the fatty acids of human adipose tissue published in *Lipids* (1), the authors make the point that they were unable to detect α-linolenic (18:3n-3) acid in any depot fats from fifteen sites on seven adult males. This is in contrast to three other studies that the authors mention as showing up to 3% of 18:3n-3 in human adipose tissue, although figures of 0.2–0.6% seem to be more common. In view of the increasing interest in ω3 fatty acids in nutrition, vascular biology and medicine (2,3), it is desirable to explain this negative finding. The authors used only OV-1 as liquid phase in gas chromatography (GC). It has long been known that 18:3n-3 acid and linoleic acid (18:2n-6) are not separated effectively on this phase (4–7). This is a general problem with n-3 and n-6 structural analogs when these also differ by one methylene-interrupted ethylenic bond. Thus a good resolution also would not be obtained between the pairs 18:3n-6 and 18:4n-3, 20:4n-6 and 20:5n-3, and 22:5n-6 and 22:6n-3, although the levels of C_{20} and C_{22} fatty acids are usually very low in human adipose tissue (8–10). Two of the latter groups of investigators used polar GC columns and show chromatograms with peaks for 18:3n-6 (γ-linolenic acid) (8,10), which also seems to be present in human adipose tissue in the 0.1–0.3% range. Modern GC with polar, open-tubular columns can resolve the polyunsaturated C_{18}, C_{20} and C_{22} fatty acids mentioned above, as well as 22:4n-6 and 22:5n-3. These polyunsaturated fatty acids should therefore all be reported, even at the low levels found in adipose tissue, as well as for other organs in which these acids are presumed to be functional and important.

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α-Linolenic Acid in Human Adipose Tissue—A Reply

Dear Sir:

We recently reported on the fatty acid compositions of fifteen adipose depots from seven adult males (1). None of the depots contained detectable levels of α–linolenic acid (18:3n-3). This finding is in contrast to at least three previous reports (2–4), each of which was discussed in our paper (1). In his letter (5), Dr. Ackman suggests that the reason for the nondetectability of this fatty acid was the type of column used for the separation of fatty acid methyl esters. Indeed, the previous studies all used a more polar gas-chromatographic column than did Calder *et al.* (1), and, as Dr. Ackman indicates, a more polar column would allow better resolution of α–linolenic acid and linoleic acid (18:2n-6). It should be pointed out, however, that the OV-1 column used is able to partially resolve these two fatty acids and that separate peaks are seen; indeed, we have reported α–linolenic acid proportions in lipids isolated from macrophages (6) and lymphocytes (7) cultured *in vitro* in the presence of α–linolenic acid and in animal diets expected to contain this fatty acid.

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ARTICLE

Characterization of Lipid Hydroperoxides Generated by Photodynamic Treatment of Leukemia Cells

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A new technique, high-performance liquid chromatography with reductive mode electrochemical detection on a mercury drop (HPLC–EC), has been used for analyzing lipid hydroperoxide (LOOH) formation in photooxidatively stressed L1210 leukemia cells. Highly specific and sensitive for peroxides (detection limits <0.5 pmol for cholesterol hydroperoxides and <50 pmol for phospholipid hydroperoxides), this approach allows different classes of LOOH to be separated and determined in minimally damaged cells. L1210 cells in serum-containing growth medium were irradiated in the presence of merocyanine 540 (MC540), a lipophilic photosensitizing dye. Lipid extracts from cells exposed to a light fluence of 0.11 J/cm^2 (which reduced clonally assessed survival by 30%) showed 12–15 well-defined peaks in HPLC–EC. None of these peaks was observed when cells were irradiated without MC540 or when dye/light-treated samples were reduced with triphenylphosphine prior to analysis. Three peaks of relatively low retention time (<12 min) were assigned to the following species by virtue of comigration with authentic standards: 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH), and 3β-hydroxycholest-5-ene-7α/7β-hydroperoxide (7α/7β-OOH). Formation of 5α-OOH and 6β-OOH (singlet oxygen adducts) was confirmed by subjecting [^{14}C]cholesterol-labeled cells to relatively high levels of photooxidation and analyzing extracted lipids by HPLC with radiochemical detection. Material represented in a major peak at 18–22 min on HPLC–EC was isolated in relatively large amounts by semipreparative HPLC and shown to contain phospholipid hydroperoxides (predominantly phosphatidylcholine species, PCOOH) according to the following criteria: (i) decay of 18–22 min peak during Ca^{2+}/phospholipase A_2 treatment, with reciprocal appearance of fatty acid hydroperoxides; (ii) reduction of peroxide during treatment with reduced glutathione and phospholipid hydroperoxide glutathione peroxidase, but not glutathione peroxidase; and (iii) comigration with PCOOH standards in thin-layer chromatography. HPLC–EC analysis revealed quantifiable amounts of PCOOH and ChOOH at a light fluence that clonally inactivated <10% of the cells, which allows for the possibility that photoperoxidative damage plays a causal role in cell killing.

***Lipids 29,* 449–459 (1994).**

Lipid peroxidation is a prominent manifestation of oxidative stress that has received considerable attention in recent years because of its cytotoxic effects and possible role in pathological conditions such as atherosclerosis, ischemia–reperfusion injury, drug-induced liver damage and carcinogenesis (1–3). Many different enzymatic and nonenzymatic agents can trigger lipid peroxidation in biological systems. Included in the former category are lipoxygenases, cyclooxygenases and cytochrome P450 reductases. Nonenzymatic lipid peroxidation can be triggered by processes such as xenobiotic autoxidation, ozone generation, ionizing radiation and photodynamic action. The surge of biological and biomedical interest in lipid peroxidation has stimulated development of powerful new techniques for separating and detecting peroxidation products and intermediates. Among these are several approaches involving high-performance liquid chromatography (HPLC) of lipid hydroperoxide (LOOH) species, with detection based on measurement of chemiluminescence (4–6), fluorescence (7) or electrochemical (EC) reduction (8–11). A great advantage of these approaches over those employing ultraviolet (UV) absorbance or radiochemical detection is that lipid hydroperoxides can be distinguished from alcohol reduction products that often accompany them in biological samples and tend to coelute with them (10,11). We have reported that HPLC–EC is an excellent approach for determining individual LOOH classes in complex mixtures of cholesterol and phospholipid hydroperoxides (12,13). Using HPLC with a renewable mercury drop electrode, we have achieved detection limits of less than 0.5 pmol for rapidly eluting cholesterol hydroperoxides (13), which exceeds the sensitivity of all other ex-

[1]This paper is based on a dissertation submitted by G.J. Bachowski in partial fulfillment of the requirements for a Ph.D. degree in Biochemistry at the Medical College of Wisconsin (Milwaukee, WI).

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Abbreviations: $AlPcS_4$, chloroaluminum phthalocyanine tetrasulfonate; ChOOH, cholesterol hydroperoxide(s); DCP, dicetylphosphate; DFO, desferrioxamine; EC, electrochemical; FAOOH, fatty acid hydroperoxide(s); FCS, fetal calf serum; GC/MS, gas chromatography/mass spectrometry; GPX, glutathione peroxidase; GSH, reduced glutathione; HPLC–EC, high-performance liquid chromatography with mercury drop electrochemical detection; HPLC–RC, high-performance liquid chromatography with radiochemical detection; LOOH, lipid hydroperoxide(s); MC540, merocyanine 540; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 6β-OOH, 3β-hydroxycholest-4-ene-6β-hydroperoxide; 7α-OOH, 3β-hydroxycholest-5-ene-7α-hydroperoxide; 7β-OOH, 3β-hydroxycholest-5-ene-7β-hydroperoxide; PBS, phosphate buffered saline (25 mM sodium phosphate, 125 mM NaCl, pH 7.4); PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide(s); PEOOH, phosphatidylethanolamine hydroperoxide(s); PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLOOH, phospholipid hydroperoxide(s); PLA_2, phospholipase A_2; POPC-OOH, hydroperoxide of 1-palmitoyl-2-oleoyl phosphatidylcholine; PLPC-OOH, hydroperoxide of 1-palmitoyl-2-linoleoyl phosphatidylcholine; TGOOH, triacylglycerol hydroperoxide(s); TLC, thin-layer chromatography; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, TPP, triphenylphosphine.

isting HPLC methods (5–7). In the work described in this paper, mercury electrode HPLC–EC has been used for analyzing the formation of several different LOOH species in photodynamically-treated leukemia cells. Preliminary findings along these lines have been published recently (13). The ultrahigh sensitivity of the HPLC–EC approach has allowed us to detect significant amounts of phospholipid and cholesterol hydroperoxides in photodamaged cells prior to any large-scale loss of viability.

MATERIALS AND METHODS

Chemicals and reagents. Cholesterol, egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), dicetylphosphate (DCP), bovine erythrocytic glutathione peroxidase (GPX), bovine pancreatic phospholipase A_2 (PLA_2), reduced glutathione (GSH), Chelex-100 (50–100 mesh), RPMI-1640 medium, insulin, transferrin, penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) were from Avanti Polar Lipids (Birmingham, AL). Hyclone Laboratories (Logan, UT) supplied the fetal calf serum (FCS). [4-^{14}C]Cholesterol (40–60 mCi/mmol in benzene) was obtained from Research Products International (Mount Prospect, IL). Desferrioxamine (DFO) was supplied by Ciba-Geigy Corp. (Suffern, NY); peroxide-free Triton X-100 by Boehringer-Mannheim Biochemicals (Indianapolis, IN); merocyanine 540 (MC540) by Eastman Kodak (Rochester, NY); and chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) by Porphyrin Products (Logan, UT). HPLC-grade chromatographic solvents were obtained from Burdick and Jackson Corp. (Muskegon, MI) or Mallinckrodt Co. (Paris, KY). Cholesterol hydroperoxide standards [3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH), 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH) and 3β-hydroxy-cholest-5-ene-7α/7β-hydroperoxide (7α/7β-OOH) mixture] were prepared and characterized as reported (12). Phospholipid hydroperoxide glutathione peroxidase (PHGPX) was prepared from rat testes as described and stored at –80°C (14,15). The purified enzyme migrated as a single band of molecular weight ~20,000 Da on sodium dodecyl sulfate–polyacrylamide electrophoresis. PHGPX activity was determined by coupled enzymatic assay, using NADPH, GSSG-reductase, GSH and photoperoxidized egg PC (14). All aqueous solutions were prepared with deionized, glass-distilled water.

Cell culture. Murine leukemia L1210 cells (ATCC CLL-219) obtained from the American Type Culture Collection (Rockville, MD) were grown in suspension culture at 37°C under a humidified atmosphere of 95% air/5% CO_2. The growth medium consisted of 1% FCS in RPMI-1640 supplemented with insulin (10 μg/mL), transferrin (5 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and sodium selenite (5 ng/mL). Cells were reseeded into fresh medium every two days. New cultures were started from frozen stock on a bimonthly basis. All experiments and determinations were carried out on cells in log-phase growth. Cell viability was assessed by 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on metabolic activity (16), and by clonogenic assay (17). Additional details are provided in Reference 18.

Protein and lipid determinations. Total protein in L1210 cells was determined by the method of Lowry *et al.* (19), using bovine serum albumin as the standard; the measured value was 6.4 ± 0.4 mg/10^8 cells (mean ± SD; n = 4).

Total cellular lipid, as determined by gravimetric analysis (18), was found to be 1.43 ± 0.06 mg/10^8 cells (mean ± SD; n = 6). Phospholipid content was measured by phosphate analysis, using a modification of the Bartlett approach (20). Samples containing ~10^7 cells were washed three times with 25 mM Hepes [*N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid)]/125 mM NaCl (pH 7.4). After the addition of 1 mM EDTA, the cell suspension was extracted with 1.6 vol of chloroform/methanol (2:1, vol/vol). The lipid extract was dried under N_2 and dissolved in 0.4 mL of 10 N H_2SO_4. After heating for 2 h at 155°C, 2 drops of 30% H_2O_2 were added, followed by 1 h of additional heating. Ammonium molybdate (0.2 mL of a 2.5% solution) and 1-amino-2-naphthol-4-sulfonic acid (0.1 mL of a 0.2% solution) were then added, along with enough water to bring the final volume to 2.5 mL. The mixture was heated at 100°C for 10 min, after which absorbance at 830 nm was recorded. An extinction coefficient of 23.2 $mM^{-1}cm^{-1}$ at this wavelength (measured with an inorganic phosphate standard) was used for quantitation. Cholesterol content was determined by reverse phase HPLC with UV (212 nm) detection (see HPLC section). Identification and quantitation of cellular cholesterol was based on reference to a cholesterol standard (retention time ~33 min). Triacylglycerol content was determined by ester bond analysis (21) after separation of triacylglycerols from phospholipids. The separation was accomplished by chromatography on 35-mg silica gel minicolumns (Alltech Associates, Deerfield, IL). Lipid samples (in 25–30 μL of isopropanol) were applied to columns that had been prewashed with isopropanol and hexane. The mobile phase solvent systems (applied sequentially by syringe) were as follows: (i) 3.0 mL of chloroform/acetic acid/methanol (97:2:1, by vol), which effected (as established with standards) the complete elution of triacylglycerols and cholesterol, leaving phospholipids behind; and (ii) 2.5 mL of methanol/chloroform/water (20:10:8, by vol), which effected the complete elution of the phospholipids. Recovered fractions were dried in a SpeedVac AS160 Concentrator (Savant Instruments, Farmingdale, NY). Triacylglycerols were treated with hydroxylamine as indicated (21); liberated fatty acyl hydroxamic acids were determined by measuring the absorbance of their ferric complexes at 530 nm. Quantitation was based on an ester group extinction coefficient of 1.02 $mM^{-1}cm^{-1}$ at this wavelength, as established with a dipalmitoyl PC standard. Isolated phospholipids were also subjected to ester bond analysis. Measured values (as wt% of total cellular lipid) were as follows: (i) phospholipid: 69.4 ± 7.5% by phosphate

assay, 72.3 ± 7.8% by ester bond assay; (ii) triacylglycerol: 27.7 ± 2.3%; (iii) cholesterol: 4.0 ± 0.4% (means ± SD; n = 3).

Radiolabeling of cells. Labeling of cholesterol in L1210 cells was accomplished by incubating the cells with unilamellar liposomes containing [4-^{14}C]cholesterol. Before incorporation into liposomes, radioactive cholesterol plus carrier (~0.3 mg total sterol) was separated from any pre-existing oxidation products by silica gel thin-layer chromatography (TLC), using heptane/ethyl acetate (1:1) as the mobile phase (22). The cholesterol zone (located by radioscanning) was removed by scraping and was eluted with chloroform/methanol (2:1, vol/vol). The extract was filtered and dried; recovered lipid (~0.8 μmol; 5–10 μCi) was mixed with 1.0 μmol of egg PC and film-dried under argon. The lipids were resuspended in deaerated, Chelex-treated phosphate buffered saline (PBS) and subjected to five freeze-thaw cycles before being passed through a 200-μm Nucleopore filter in a lipid extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada) (23). Cells were centrifuged, resuspended to a concentration of ~10^7/mL in 10% heat-inactivated FCS/RPMI medium, and mixed with radiolabeled liposomes at a ratio of cellular to liposomal cholesterol of ~4:1 (mol/mol). After 15 h of incubation at 37°C under sterile conditions, the cells were centrifuged, washed extensively with 1% heat-inactivated FCS/RPMI medium to remove the liposomes and resuspended in this medium. A typical incubation as described resulted in a 30–35% exchange (transfer) of [^{14}C]cholesterol.

Photooxidation reactions. Immediately before irradiation, unlabeled or [^{14}C]cholesterol-labeled L1210 cells were centrifuged and resuspended to a concentration of 1.0–3.0 x 10^7/mL in serum-free RPMI medium. The cells were incubated with sensitizer (25 μM MC540) for 30 min in the dark, after which 10-mL aliquots were transferred to 25-cm^2 culture flasks and irradiated on a translucent platform over a twin-bank of cool white 40-W fluorescent tubes. Incident light intensity (fluence rate) at the platform surface was ~7.5 W/m^2, as measured with a radiometer (Yellow Springs Instruments, Yellow Springs, OH). Temperature of the cell suspension was maintained at 25 ± 2°C. After different periods of irradiation (typically no longer than 4 min, corresponding to a fluence of 0.18 J/cm^2), the suspensions were centrifuged; each cell pellet was resuspended in 0.5 mL of PBS containing 1.0 mM EDTA. After extraction with chloroform/methanol (2:1, vol/vol) and drying under argon, the lipid fraction was analyzed iodometrically for peroxide content (see below) and then subjected to analytical HPLC.

For preparing relatively large amounts of cellular LOOH for semipreparative HPLC, the above procedure was scaled-up and $AlPcS_4$ (a more potent singlet oxygen generator; Ref. 24) was used instead of MC540. Typically, 50-mL aliquots of cells (1.0 × 10^7/mL in RPMI medium) were transferred to 165-cm^2 flasks, sensitized with 10 μM $AlPcS_4$, and irradiated for 1 h under a quartz-halogen source at 25°C. The fluence rate was ~2.3 kW/m^2. After irradiation, the cells were pelleted and lipids extracted as described above. Extracts from six of these preparations (corresponding to ~26 mg of lipid from ~3×10^9 cells) were combined and dried under argon. Recovered material was dissolved in 220 μL of isopropanol, which comprised a single injection on semipreparative HPLC.

Iodometric analysis. The LOOH content of standards and experimental samples was determined by iodometric assay, as described (25,26). Hydroperoxides were reduced anaerobically in the presence of excess iodide, with stoichiometric formation of triiodide. The latter was measured spectrophotometrically at 353 nm, quantitation being based on an extinction coefficient of 22.5 mM^{-1}cm^{-1}.

TLC. Cholesterol and phospholipid hydroperoxides were chromatographed on Silica Gel 60 plates of 0.25 mm thickness (EM Science, Cherry Hill, NJ). The mobile phase for cholesterol hydroperoxides was benzene/ethyl acetate (1:1, vol/vol), whereas that for phospholipid hydroperoxides was chloroform/methanol/water (75:25:4, by vol). Peroxides were visualized by spraying with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD). Other details were as described (22,27,28).

HPLC. Liquid chromatography was accomplished with an Isco integrated HPLC system (Isco Inc., Lincoln, NE). Analytical reverse-phase HPLC–EC was carried out at room temperature, using a C18 Ultrasphere column (4.6 × 150 mm; 5 μm particles) from Beckman Instruments (San Ramon, CA) and C18 guard column (4.6 × 45 mm) from Alltech Associates (Deerfield, IL). Except where indicated, the premixed mobile phase consisted of (by vol) 79.5% methanol, 11.5% acetonitrile and 9.0% of an aqueous solution containing 10 mM ammonium acetate and 0.25 mM sodium perchlorate (pH 5.0). Delivered isocratically at a flow rate of 2.0 mL/min, the mobile phase was sparged continuously with high-purity argon that was passed first through an OMI-1 oxygen scrubber from Supelco Inc. (Bellefonte, PA) and then through a presaturating mobile phase scrubber. The column effluent was passed through two detectors in series, an Isco V^4 variable wavelength detector and an EG&G-Princeton Model 420 electrochemical detector with a renewable mercury drop electrode system (Princeton, NJ). A new mercury drop was dispensed and equilibrated for 5–10 min before each injection of analyte; background noise typically dropped to a satisfactory low level during this period. The electrode potential was set at –300 mV vs. an Ag/AgCl reference. Samples were dissolved in isopropanol; immediately before injection, the solutions were sparged for 2–3 min with high-purity helium to minimize O_2 entry into the system. The injection volume for all samples was 10 μL. Data collection, storage and manipulation was accomplished with an on-line AT clone and Isco ChemResearch software. Other details about the methodology have been reported elsewhere (12,13,29). Identification and quantitation of various hydroperoxides detected in cell samples were based on retention times and EC responses of standards, e.g. 5α-OOH, 6β-OOH, 7α/7β-OOH, POPC-OOH, and PLPC-OOH. 5α-OOH and 7α/7β-OOH were found to have the same EC response, which was ~25% higher than that of 6β-OOH; POPC-OOH and PLPC-OOH also

had the same response, but it was about half that of 5α-OOH or 7α/7β-OOH (13).

Semipreparative HPLC–EC was carried out on a Beckman Ultrasphere C18 column (10.0 x 250 mm; 5-μm particles) with an Alltech guard column (4.6 × 45 mm). In this case, the mobile phase consisted of 83% methanol, 10% acetonitrile and 7% aqueous solution containing 10 mM ammonium acetate and 0.25 mM sodium perchlorate (pH 5.0); flow rate was typically 4.0 mL/min.

A Baker silica column (4.6 × 250 mm; 5-μm particles) was used for normal-phase HPLC–RC of lipid extracts from photooxidized [^{14}C]cholesterol-labeled cells. The column was eluted with 97% hexane/3% isopropanol at a flow rate of 1.25 mL/min. ^{14}C-Labeled analytes were detected with a Radiomatic A100 Flow-One\Beta detector equipped with a 2.5-mL flow cell (Radiomatic Instruments, Tampa, FL).

Enzymatic characterization of isolated peroxide fractions. The 18–22 min fraction isolated by semipreparative HPLC–EC was treated with GSH and selenoperoxidases as follows. Samples of the 18–22 min material (~5 nmol of iodometrically determined LOOH) were solubilized in 0.5 mL of Chelex-treated PBS containing 0.1% Triton X-100 and 20 μM DFO. (DFO was included to prevent any iron-catalyzed LOOH degradation.) After addition of GSH alone (1 mM), GSH plus PHGPX (0.1 U/mL), or GSH plus GPX (0.5 U/mL), the mixtures were incubated for 30 min at 37°C, after which lipids were extracted and analyzed by PLOOH-resolving TLC (see above), TMPD being used for peroxide detection.

Phospholipase-A_2 treatment of the 18–22 min fraction was carried out as follows. Samples containing ~5 nmol of total LOOH were film-dried in acid-washed tubes, then vortexed into 0.5 mL of 50 mM Chelex-treated Tris-HCl (pH 7.4) containing 20 μM DFO. After addition of $CaCl_2$ (5 mM) and PLA_2 (60 U/mL), the mixtures were re-vortexed and incubated for periods of up to 1 h at 37°C. A control containing everything except PLA_2 was run alongside. After incubation, lipids were extracted, dried, dissolved in isopropanol and analyzed by HPLC–EC, using the following mobile phase: 85% methanol/9% acetonitrile/6% aqueous solution containing ammonium acetate and sodium perchlorate. The lower polarity of this eluant compared with that used for total LOOH (see preceding section) sharpened the parent peroxide peak by decreasing its retention time, thus permitting hydrolytic decay to be measured with greater sensitivity. For monitoring the formation of fatty acid hydroperoxide (FAOOH) products, a different mobile phase was used, namely a 50% methanol/27.5% acetonitrile/22.5% aqueous solution, which satisfactorily separated FAOOH species without solvent front interference. Identification and quantitation of the FAOOH was based on retention times and EC responses of peroxidized linoleic acid and arachidonic acid standards. The latter were typically prepared by irradiating the fatty acids, 100 mM in chloroform/methanol (1:1, vol/vol), with a quartz-halogen source for 3 h in the presence of 10 μM $AlPcS_4$ at 10°C. After drying, resuspending in PBS/EDTA, extracting and redrying, the standards were determined iodometrically and then chromatographed alongside the unknowns.

RESULTS

HPLC–RC of lipid extracts from photoperoxidized [^{14}C]cholesterol-labeled cells. Total lipid extracted from [4-^{14}C]cholesterol-labeled L1210 cells before and after irradiation in the presence of MC540 was subjected to HPLC–RC analysis. The unirradiated sample (Fig. 1A) shows a major peak of radioactivity at ~5 min, assigned as cholesterol; and a lesser peak at ~3 min, tentatively assigned as cholesteryl ester(s). The apparent transesterification may have been catalyzed by a cellular acyl-CoA/cholesterol acyltransferase, since the serum used in the incubation mixture was heat-treated to inactivate lecithin/cholesterol acyltransferase. The irradiated sample (Fig. 1B) shows at least three distinct product peaks, two of which have been assigned on the basis of matching retention times of standards: 6β-OOH (9.5 min) and 5α-OOH (13.0 min). Product yields relative to total injected radioactivity were ~1.0 and ~2.3%, respectively. Identification of 5α-OOH and 6β-OOH provides unambiguous evidence for singlet oxygen ($^1O_2$) involvement in the photooxidative process (30,31). This is consistent with previous results showing $^1O_2$ intermediacy in

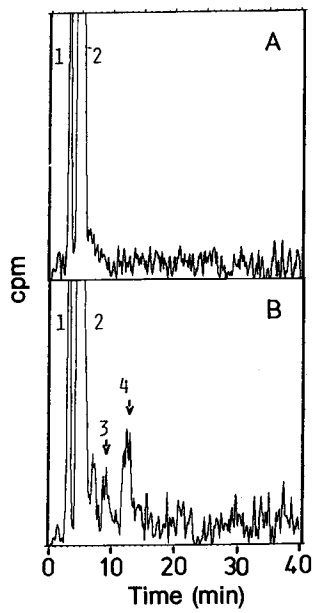

FIG. 1. Normal-phase high-performance liquid chromatography–radiochemical detection (HPLC–RC) of cholesterol hydroperoxides from photooxidized [^{14}C]cholesterol-labeled L1210 cells. Cells (~3.0 × 10^7/mL in medium containing 10% heat-inactivated serum) were radiolabeled by incubating with egg phosphatidylcholine/cholesterol (10:8, mol/mol) liposomes containing ~10 μCi of thin-layer chromatography-purified [4-^{14}C]cholesterol. After washing and resuspending in 1% serum/RPMI medium, the cells were sensitized with 25 μM merocyanine 540 and analyzed by HPLC–RC before (A) and after (B) irradiating for 90 min (fluence ~4.1 J/cm^2). Samples for HPLC–RC contained ~60 μg of total lipid. Peak assignments: 1, unidentified component(s); 2, cholesterol; 3, 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH); 4, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH). Retention times of separately chromatographed standards (determined by A_{212}) were as follows: 6β-OOH, 9.5 min (arrow); 5α-OOH, 13.0 min (arrow).

model systems, e.g., MC540–sensitized liposomes or erythrocyte membranes (26,32). It should be pointed out that in order to detect product signals by HPLC–RC (cf. Fig. 1B), it was necessary to irradiate cells to extreme limits, killing >99% of the population. The fact that MC540 is a rather modest $^1O_2$ generator (33) and undergoes self-sensitized photobleaching (32), added to the difficulty of measuring cellular photoproducts by HPLC–RC. To improve the sensitivity of LOOH measurement, we switched to reverse-phase HPLC with EC detection. As demonstrated recently (13), subpicomole detection limits can be achieved for ChOOH standards with this approach.

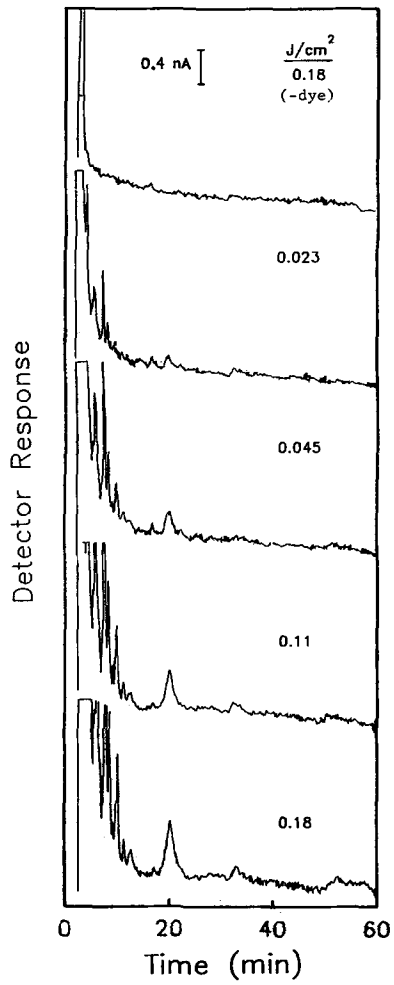

FIG. 2. Reverse-phase HPLC–EC (electrochemical detection) of lipid hydroperoxides from photooxidized L1210 cells. Cells (1.0×10^7/mL in 1% serum/RPMI medium) were irradiated in the absence of sensitizer (top scan) or presence of 25 μM merocyanine 540; at the indicated fluences (J/cm^2) of delivered light, samples containing ~290 μg of total lipid were removed for chromatographic analysis. (The smallest fluence shown, 0.023 J/cm^2, represents 0.5 min of irradiation at a fluence rate of 7.5 W/m^2.) Iodometrically-determined hydroperoxide content of the samples (viewing scans from top to bottom) was as follows: <0.2 nmol; <0.5 nmol; 0.5 nmol; 1.3 nmol; 2.5 nmol. Full-scale detector sensitivity was 2.0 nA for each determination. See Figure 1 for abbreviations.

HPLC–EC of lipid extracts from photoperoxidized cells. HPLC–EC profiles of lipid extracts from cells that had been subjected to modest levels of MC540-sensitized photooxidation are shown in Figure 2. At the lowest delivered light fluence, 0.023 J/cm^2, one can see discrete EC peaks distributed over a 60-min retention time range. The intensity of these peaks increases with increasing fluence up to 0.18 J/cm^2 with no indication of any significant shift in distribution. At least ten major peaks can be distinguished at the 0.18 J/cm^2 level: a tight cluster of five or six peaks in the 5–20-min range, one prominent broad peak centered at ~20 min, and three lesser others at ~33, ~53 and ~57 min. None of these peaks was detected in cells exposed to a 0.18 J/cm^2 fluence in the absence of MC540 (Fig. 2) or in MC540-sensitized cells kept in the dark. Thus, there was no significant background level of species giving EC signals; moreover, these species could not have been generated by light alone or light acting on some endogenous sensitizing agent. Viability assays on cell samples from the Figure 2 experiment showed that cell killing was relatively modest for light fluences that produced substantial EC signals. For example, at 0.045 J/cm^2, cell survival measured by clonal assay was >90% and by MTT assay >95% (Table 1). Even at the highest fluence used in this experiment (0.18 J/cm^2), survival was >10%. Thus, in contrast to radio detection, EC detection allowed us to measure significant levels of photoproducts in minimally damaged cells.

HPLC profiles observed with EC and UV (212 nm) detection were compared for lipid extracts from nontreated and MC540/light-treated cells. As shown in Figure 3, the control (no dye/light) sample exhibited several UV peaks over a 60-min retention time range, but no significant EC peaks. After photooxidation (0.18 J/cm^2 fluence), a characteristic EC profile was observed, whereas the UV profile remained essentially unchanged. When a sample from photooxidized cells was treated with triphenylphosphine (TPP) prior to HPLC, all EC peaks disappeared; once again, the UV profile was unaffected

TABLE 1

Viability of Dye/Light-Treated L1210 Cells[a]

Fluence (J/cm^2)	Clonal assay (viable cells, %)[b]	MTT assay (viable cells, %)[c]
0	100 ± 3	100 ± 4
0.045	91 ± 6	96 ± 6
0.113	65 ± 8	75 ± 1
0.180	12 ± 2	65 ± 2
0.180 (control)[d]	99 ± 6	96 ± 3

[a]Cells (1.0×10^7/mL) were irradiated at 25°C in the presence of 25 μM merocyamine 540 (MC540), except where indicated. Samples are from the experiment described in Figure 2.
[b]Clonal survival is represented as a percentage of colonies formed relative to nonirradiated controls; values are means ± SD (n = 3).
[c]Absorbance at 570 nm in 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is represented as a percentage of the absorbance produced by nonirradiated control cells; means ± deviation of values from two determinations.
[d]Cells irradiated in the absence of MC540.

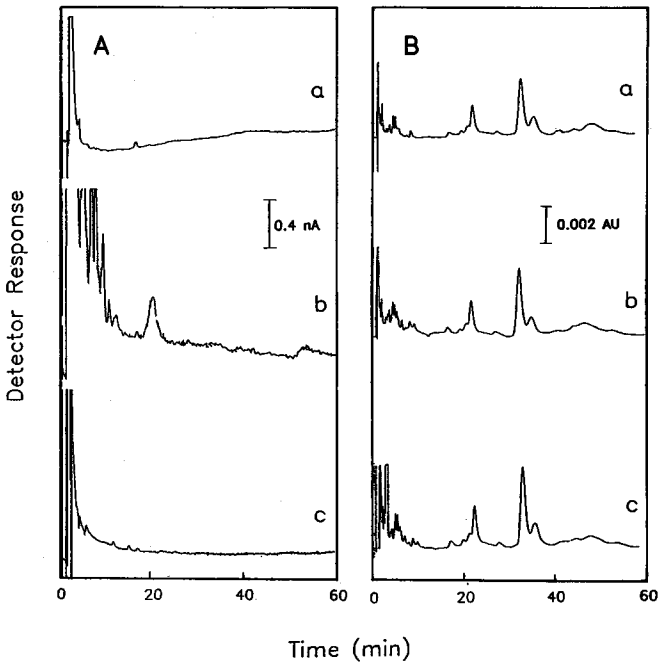

FIG. 3. HPLC analysis of lipid extracts from photoperoxidized L1210 cells. Comparison of profiles obtained with electrochemical detection (panel A) and ultraviolet (A_{212}) detection (panel B). Injected samples, each containing ~290 µg of total lipid, were as follows: (a) extract from nontreated cells; (b) extract from cells exposed to a 0.18 J/cm^2 light fluence in the presence of 25 µM MC540 (~2.5 nmol of total lipid hydroperoxide (LOOH) in injected sample); and (c) extract from photoperoxidized cells (as in b) after treatment with 10 nmol of triphenylphosphine. See Figure 2 for other abbreviation.

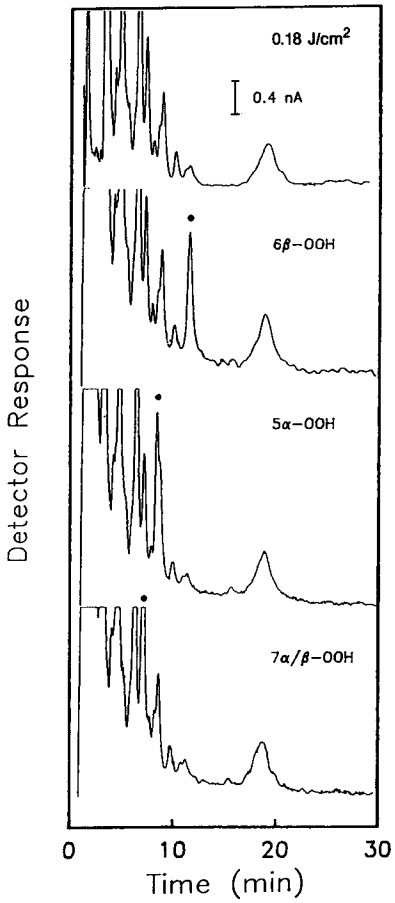

FIG. 4. HPLC–EC profiles of photoperoxidized L1210 cell lipid extracts that were spiked with selected cholesterol hydroperoxide standards. Lipid extracts were spiked with authentic 6β-OOH (11.5 min), 5α-OOH (8.4 min) or 3β-hydroxycholest-5-ene-7α-hydroperoxide/3β-hydroxycholest-5-ene-7β-hydroperoxide (7α/7β-OOH) (7.2 min), as indicated. Injected samples contained ~290 µg of lipid, ~2.5 nmol of LOOH, and 40 pmol of hydroperoxide standard (except for top chromatogram). Peaks corresponding to the added standards are notated by dots. See Figures 1–3 for abbreviations.

except for the appearance of a new peak at ~3 min representing TPP (Fig. 3). TPP is an oxygen-abstracting agent that can effectively reduce hydroperoxides to hydroxides (34). Since the latter are EC-silent, this evidence is consistent with the notion that species giving EC signals are peroxides. As demonstrated previously for simpler (noncellular) systems (12), the contrast in EC and UV responses after reductive treatment highlights the selectivity of EC for peroxide detection under the redox conditions chosen.

Assignment of cholesterol hydroperoxides in HPLC–EC profiles. Realizing that MC540 localizes in the plasma membrane, where most of the cellular cholesterol is located, and that MC540 can sensitize cholesterol peroxidation (Fig. 1), we suspected that some of the EC peaks observed in Figures 2 and 3 (retention times <15 min) represent ChOOH products. As a first attempt at identifying these species, we spiked photooxidized samples with individual standards (5α-OOH, 6β-OOH or 7α/7β-OOH mixture) and located matching positions on HPLC–EC profiles. As shown in Figure 4, a sample peak corresponding to unresolved 7α/7β-OOH (7.2 min) is sharp and distinct. The peak corresponding to 5α-OOH (8.4 min) appears to overlap with that of a more abundant (unidentified) species. Similarly, 6β-OOH appears to be unresolved from another minor species. Whether this might be 6α-OOH, which was shown to be less abundant than the 6β epimer in model systems (12), is presently unknown. Quantitation of cellular 5α-OOH, 6β-OOH and 7α/7β-OOH (collectively referred to as ChOOH; see Table 3) was accomplished by spiking samples with increasing amounts of the respective standards, integrating, and extrapolating to zero addition.

To strengthen our argument about ChOOH identities, we isolated relatively large amounts of these species by semipreparative HPLC–EC and subjected them to alternative TLC analysis along with appropriate standards. As shown in Figure 5 (lane c), a fraction believed to contain 7α/7β-OOH on the basis of spiking results (Fig. 4) actually contained one major peroxide species, which comigrated with authentic 7α/7β-OOH (lane a). Material from the fraction corresponding to 5a-OOH was found to be quite contaminated with other peroxides, as expected (Fig. 5, lane e). However, visualization of a faint spot at the same position as authentic 5α-OOH (lane b) confirms that at least some of this species was present in

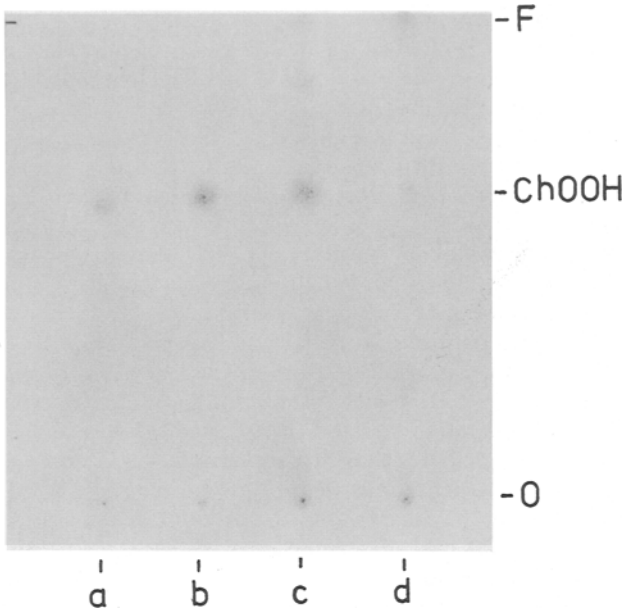

FIG. 5. Thin-layer chromatogram of cholesterol hydroperoxides (ChOOH). Fractions suspected of containing ChOOH (5α-OOH, 6β-OOH and 7α/7β-OOH) were isolated by semipreparative HPLC–EC of lipid extracts from large-scale preparations of photooxidized L1210 cells (see Materials and Methods section). Material from these fractions was analyzed by silica gel thin-layer chromatography (TLC), using benzene/ethyl acetate (1:1, vol/vol) as the mobile phase. Hydroperoxides were visualized by spraying with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Sample lanes were as follows: (a) 7α/7β-OOH standard mixture; (b) 5α-OOH standard; (c) HPLC fraction believed to contain 7α/7β-OOH; and (d) HPLC fraction believed to contain 5a-OOH. See Figures 1–4 for other abbreviations; O indicates origin; F indicates solvent front.

mildly photooxidized cells. Although ChOOH species do not separate well from one another on TLC (Fig. 5), diol reduction products do separate satisfactorily (22,28). However, it was not possible to identify and differentiate cell-derived 7α/7β-OOH and 5α-OOH on this basis because sample material was limited and the detection method, spraying with 50% sulfuric acid (27), is not sensitive enough.

Assignment of phospholipid hydroperoxides in HPLC–EC profiles. We have reported elsewhere (13) that photochemically-generated hydroperoxides of two synthetic phospholipids, POPC and PLPC, elute as broad peaks on HPLC–EC, their average retention times being longer than those of ChOOH. These time ranges, particularly the POPC–OOH range, fall close to that of the broad peak appearing at 18–22 min in the present study (Figs. 2 and 3). Suspecting, therefore, that the 18–22-min peak might represent phospholipid hydroperoxides, we used semipreparative HPLC–EC to isolate this material in relatively large amounts for further study. Although this preparation was relatively pure in terms of (putative) hydroperoxide type, it may have contained large amounts of other (EC-silent) species, e.g., unoxidized lipids, making identification by spectroscopic methods, e.g., gas chromatography/mass spectrometry (GC/MS), difficult, if not impossible. We selected two different biochemical approaches as alternatives.

To confirm that the 18–22-min fraction contained hydroperoxides and that they were derived from cellular phospholipids, we treated samples with GSH and two different selenoperoxidases, GPX and PHGPX. PHGPX can catalyze the reduction of membrane lipid hydroperoxides to EC-silent alcohols (35). In addition to PLOOH, the enzyme can act on ChOOH and TGOOH (35,36). GPX can reduce relatively polar peroxides such as H_2O_2 and FAOOH, but does not act on LOOH, whether membrane-bound or solubilized (37). TLC analysis (Fig. 6, lane b) indicated that the 18–22-min fraction contained one major TMPD-reactive compound with mobility matching that of an egg PCOOH standard plus a trace of material migrating as PEOOH (see also Ref. 13). A 30-min incubation of the 18–22-min fraction in the presence of Triton X-100, GSH, PHGPX and DFO resulted in complete disappearance of TMPD-positive spots, including the zone observed at the solvent front (Fig. 6, lane d). (We determined that the latter was generated as an ar-

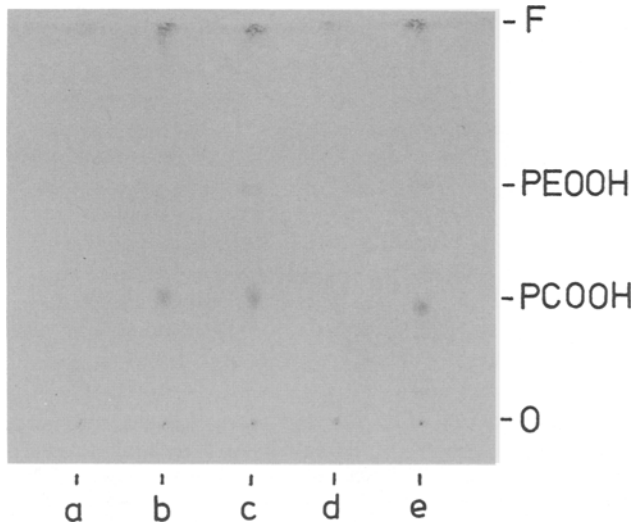

FIG. 6. Thin-layer chromatogram of 18–22-min fraction after treatment with glutathione and selenoperoxidases. Samples of 18–22 min HPLC fraction from a lipid extract of photooxidized L1210 cells were solubilized in Chelex-treated phosphate buffered saline (25 mM sodium phosphate, 125 mM NaCl, pH 7.4) containing 0.1% Triton X-100, 20 μM desferrioxamine (DFO) and 3.0 mM reduced glutathione. Lipids were extracted and analyzed by TLC before incubation (lane b) and after incubation for 30 min at 37°C in the absence of selenoperoxidase (lane c), presence of 0.1 U/mL phospholipid hydroperoxide glutathione peroxidase (lane d), or presence of 0.5 U/mL glutathione peroxidase (lane e). Lane a represents an extract of reaction mixture containing all but the 18–22-min material. Starting material applied in lanes b–e contained ~1.0 nmol of LOOH. Phosphatidylcholine hydroperoxides (PCOOH) and phosphatidylethanolamine hydroperoxides denote positions of photoperoxidized egg phosphatidylcholine and egg phosphatidylethanolamine standards (spots not shown). See Figures 1–5 for other abbreviations.

tifact during sample incubation at 37°C.) No loss of TMPD reactivity was observed when samples were incubated with GSH alone (lane c) or GSH plus GPX (lane e); five-times as many units of GPX were used, yet it was unreactive relative to PHGPX. These results support our contention that the major component observed in the 18–22-min fraction is a phospholipid hydroperoxide, most likely PCOOH.

Additional evidence that the 18–22-min hydroperoxide is derived from phospholipid (mainly PC) was sought by treating samples with Ca^{2+}-dependent PLA_2 to specifically cleave the *sn*-2 fatty acyl groups. Incubation with Ca^{2+}/PLA_2 resulted in a partial decay of the 18–22 min peak on HPLC–EC (Fig. 7A) and appearance of relatively high mobility products (Fig. 7B). Detection of the latter required a separate HPLC–EC run with a more polar mobile phase than used for the parent material. The chosen eluant effected a satisfactory separation of hydrolysis products from the solvent front; a partially resolved doublet appeared at ~4 min and a much smaller shoulder at ~3 min (Fig. 7B). Neither of these was observed in a control incubated with Ca^{2+} alone. Both disappeared when a sample was treated with triphenylphosphine subsequent to Ca^{2+}/PLA_2 (not shown). The EC peaks observed in Figure 7B are ascribed to FAOOH on the basis of matching retention times of photoperoxidized fatty acid standards (not shown), e.g., oleic acid hydroperoxide (~4 min) and linoleic or arachidonic acid hydroperoxide (~3 min). There was a 46% decrease in the amount of starting material after 30 min of incubation with PLA_2 (60 U/mL), and a 56% decrease after an additional 30 min following a second dose of enzyme (Table 2). The observed conservation of total hydroperoxide during Ca^{2+}/PLA_2 treatment (Table 2) indicates that nonenzymatic losses were minor and that all peroxide species were properly accounted for and quantitated. If all hydroperoxides in the 18–22-min fraction existed as PLOOH and occupied the *sn*-2 position of PLOOH, they should have been completely released as FAOOH. That a <60% conversion was actually observed is attributed to the nonideal physical state of the substrate during exposure to PLA_2. Under the conditions used, substrate lipid probably existed in multilamellar form, which could have prevented complete accessibility of peroxidized groups to PLA_2, an enzyme that functions most efficiently on a monolayer or bilayer surface (38). That EC-responsive material in the 18–22-min fraction was converted stoichiometrically, albeit incompletely, to EC-responsive products

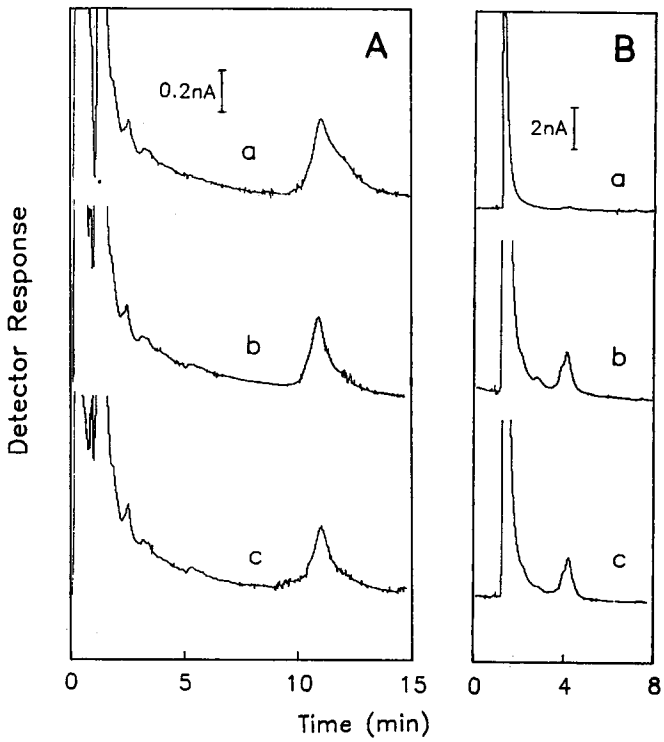

FIG. 7. HPLC–EC of the 18–22-min fraction after Ca^{2+}/phospholipase A_2 treatment. The 18–22-min fraction, isolated by semipreparative HPLC–EC of total lipid extracted from photooxidized cells, was incubated in the presence of Ca^{2+} (5 mM), PLA_2 (up to 120 U/mL) and DFO (20 μM) in 50 mM Tris buffer (pH 7.4) at 37°C. The following samples were chromatographed: (a) a 60-min control sample without PLA_2; (b) material treated with a single dose of PLA_2 (60 U/mL) and incubated for 30 min; (c) material treated with two doses of PLA_2 (60 U/mL), one each at 0 min and 30 min, and incubated for 60 min total. (A) HPLC–EC of the parent material; (B) HPLC–EC of hydrolysis products. Mobile phase in (A) consisted of 85% methanol, 9% acetonitrile, and 6% aqueous solution (pH 5.0) containing 10 mM ammonium acetate and 0.25 mM sodium perchlorate. Retention time for suspected PCOOH is 10–13 min in this system. Mobile phase in (B) consisted of 50% methanol, 27.5% acetonitrile, and 22.5% aqueous solution (see above). The peak at ~4 min in scans b and c (panel B) matches retention of oleic acid hydroperoxide, whereas the shoulder at ~3 min matches retention of arachidonic acid or linoleic acid hydroperoxide. Both of these areas were integrated for quantitation of fatty acid hydroperoxide in Ca^{2+}/PLA_2-treated samples. Integrated values for substrate and product peaks are shown in Table 2. See Figures 1, 2 and 6 for other abbreviations.

TABLE 2

Hydroperoxide Distribution After Ca^{2+}/Phospholipase A_2 (PLA_2) Treatment of the 18–22-min Fraction[a]

Sample	PLOOH (nmol)	FAOOH (nmol)	Total hydroperoxide (nmol)
Control	0.28 ± 0.04[b]	0	0.28 ± 0.04
Ca^{2+}/PLA_2 (30 min)	0.15 ± 0.01	0.16 ± 0.01	0.31 ± 0.02
Ca^{2+}/PLA_2 (60 min)	0.13 ± 0.01	0.18 ± 0.05	0.31 ± 0.06

[a]Analytical high-performance liquid chromatography with mercury drop electrochemical detection data are from the experiment shown in Figure 7. Reaction mixtures included material from the 18–22-min fraction (total iodometric LOOH ~3 μM), 5 mM $CaCl_2$, and 20 μM DFO in 50 mM Tris buffer (pH 7.4) at 37°C. Mixtures were incubated for (i) 30 min in the absence of PLA_2 (control); (ii) 30 min in the presence of PLA_2 (60 U/mL); or (iii) 60 min in the presence of PLA_2 (60 U/mL added at 0 min and again at 30 min). Suspected phospholipid hydroperoxides (PLOOH) in the 18–22-min fraction were quantified by relating their integrated peak area to that of a PLPC–OOH standard; fatty acid hydroperoxides (FAOOH) were determined by relating peak areas to those of arachidonic and linoleic acid hydroperoxide standards.
[b]Values are means ± deviation of two determinations.

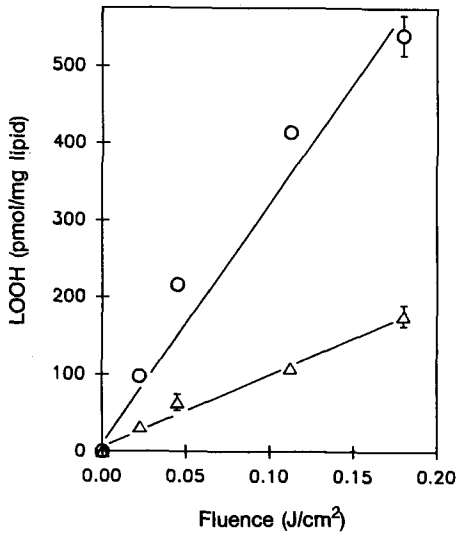

FIG. 8. Accumulation of ChOOH and PCOOH as a function of light fluence. L1210 cells (1.0×10^7/mL) were irradiated in the presence of 25 μM MC540. At the indicated fluence values, lipids were extracted and analyzed by HPLC–EC (~280 μg of total lipid per injected sample). Total ChOOH content (Δ) in each sample is the sum of peak areas for 5α-OOH, 6β-OOH and 7α/7β-OOH. Due to overlap with other species (cf. Fig. 4), 5α-OOH and 6β-OOH were each determined by measuring total peak areas after spiking samples with increasing known amounts of a standard, and extrapolating to zero addition. Total PCOOH content (○) in each sample was determined from the area of the 18–22-min peak. Data points with error bars are means ± SD (n = 4). See Figures 1–6 for abbreviations.

with the mobility of FAOOH is added evidence that the parent material was PLOOH, mainly PCOOH.

Quantitation of cholesterol and PCOOH. A plot of HPLC–EC-determined ChOOH and PCOOH levels as a function of light fluence for MC540-sensitized L1210 cells is shown in Figure 8. As can be seen, each population accumulated in linear fashion over the fluence range used, PCOOH more rapidly than ChOOH by a factor of ~3.5. This is consistent with the fact that cholesterol contains only one double bond, whereas cellular PC would average more than one double bond at the *sn*-2 fatty acyl position, making them intrinsically more oxidizable. It would be difficult to predict the relative rates of PCOOH and ChOOH accumulation in this system, since the actual content of unsaturated fatty acyl groups in PC is unknown. Moreover, additional information (also not available) would be required, making this comparison quite complex, e.g., (i) location of membrane-bound sensitizer relative to PC and cholesterol; (ii) O_2 level in the local environment of PC relative to cholesterol; and (iii) possible differences in the metabolic turnover of PCOOH and ChOOH. Table 3 shows yields of photochemically generated ChOOH and PCOOH expressed as percentages of overall LOOH and as percentages of total lipid in each respective class. As can be seen, ChOOH accounted for 2–3 mol% of the total LOOH over the fluence range used. This is reasonably close to the mol fraction of cholesterol relative to total lipid in our cells (see Materials and Methods section), suggesting that cholesterol and other lipids are similarly accessible to photooxidants. As also shown in Table 3, PCOOH in the 18–22-min fraction accounted for 7–10 mol% of the total LOOH generated at different stages of photooxidation. This value is not far different from the mol% of PC relative to total lipid (39), again suggesting an even distribution of oxidant(s) among reactive lipids. Over a fluence range of 0.045 to 0.18 J/cm^2, ChOOH content expressed as mol% of parent lipid increased from 0.05 to 0.13; PCOOH content expressed as mol% of PC increased by the same amount (Table 3). These data also imply similar accessibility of PC and cholesterol to MC540/light-derived oxidant(s).

TABLE 3

Relative Yields of Lipid Hydroperoxides in MC540/Light-Treated Cells[a]

Fluence (J/cm^2)	Total LOOH nmol/10^8 cells[b]	ChOOH (mol %)[c]		PCOOH (mol %)[d]	
		% LOOH	% Ch	% LOOH	% PC
0	<0.2	—	—	—	—
0.045	0.5	3.0	0.05	10.0	0.05
0.11	1.3	2.2	0.08	7.7	0.10
0.18	2.5	2.5	0.13	8.4	0.13

[a]Cells (1.0×10^7/mL) were exposed to the indicated light fluences in the presence of 25 μM merocyamine 540 (MC540) (see Materials and Methods section).
[b]Total lipid hydroperoxide (LOOH) content was determined by iodometric assay.
[c]Cholesterol hydroperoxide (ChOOH) content of cells was determined by high performance liquid chromatography–electrochemical detection (HPLC–EC). Values are expressed relative to total LOOH and total cholesterol. Cholesterol (Ch) content determined by HPLC with ultraviolet detection was 147 ± 15 nmol/10^8 cells; this translates to 4.0 ± 0.4% (w/w) of total lipid, based on a gravimetrically determined lipid content of 1.42 ± 0.06 mg/10^8 cells.
[d]Phosphatidylcholine hydroperoxide (PCOOH) content of cells was determined by HPLC–EC. The value used for phosphatidylcholine (PC) content was 412 nmol/10^8 cells (Ref. 40).

DISCUSSION

Increasing interest in the cytopathological effects of lipid peroxidation has stimulated the development of highly sensitive and selective approaches for assessing this process. Approaches involving direct measurement of lipid hydroperoxides are especially important because these species can play a central role in cytotoxicity, e.g., by directly perturbing membrane structure and function or by acting as "nuclei" for free radical reactions that exacerbate overall damage. Moreover, identification of certain LOOH (most notably those in the ChOOH family) can provide valuable mechanistic information, e.g., whether a reaction is $^1O_2$-mediated or free radical-mediated. HPLC, with some type of high sensitivity/specificity detection mode such as chemiluminescence, fluorescence or reductive electrochemistry, has become the method of choice for analyzing different LOOH in complex mixtures. Each of these modes has advantages and disadvantages, but it is becoming increasingly clear that EC detection provides the best option in terms of (i) overall cost; (ii) ease of operation and maintenance; and (iii) fewer possibilities of artifactual signals. The latter are less likely to arise in EC because post-column mixing reactions are not required for generating signals. HPLC–EC with a glassy carbon indicator electrode has been used for analyzing PLOOH (8–10) and FAOOH (11). Subsequent work carried out in this laboratory showed that this approach is also suitable for ChOOH products, with detection limits of 25–30 pmol being achieved (12). More recently, we discovered that a renewable mercury drop electrode has several advantages over the glassy carbon electrode, most impressive of which is the 100–200-fold decrease in detection limit for ChOOH. HPLC with mercury electrode EC can be run under conditions that permit baseline separation of ChOOH (5α-OOH, 6β-OOH, 7α/7β-OOH), not only from one another, but also from various PLOOH (e.g., POPC-OOH, PLPC-OOH, egg PCOOH). Preliminary studies (13) demonstrated the feasibility of using this method for monitoring LOOH accumulation in oxidatively stressed cells, e.g., L1210 cells exposed to photodynamic action.

Studying MC540/light-treated cells more extensively in the present work, we have identified and quantified several LOOH species arising during early, minimally lethal, stages of oxidative injury. These include (i) 5α-OOH and 6β-OOH, characteristic products of $^1O_2$ attack on cholesterol (30); (ii) the 7α/7β-OOH epimers, presumed from earlier work (26) to be rearrangement products of 5α-OOH; and (iii) a PLOOH mixture consisting almost exclusively of PCOOH. The extremely high sensitivity of mercury electrode EC permitted measurement of these species at levels that were far below the UV absorbance detection limits at 212 nm (ChOOH) or 233 nm (PLOOH) (13). Although several peaks were observed in the UV detection mode (Fig. 3), these had nothing to do with peroxides, since samples analyzed before and after photooxidation, and also after photooxidation followed by TPP treatment, all had the same UV profile. By contrast, EC peaks appeared only in photooxidized samples and disappeared after incubation with TPP, consistent with what one would expect for reducible LOOH. Definitive identification of the LOOH species, e.g., by spectroscopic techniques such as GC/MS or high resolution nuclear magnetic resonance, was not possible due to low yields, peroxide microheterogeneity, and the presence of co-eluting, nonperoxide contaminants. Large-scale purifications for this purpose were beyond the scope of this work. However, we were able to establish LOOH class identity in other ways, e.g., by using matching standards on HPLC and TLC. In the case of the 18–22-min family, PLOOH (PCOOH) identity was confirmed by demonstrating (i) reactivity with GSH/PHGPX, but not GSH/GPX; and (ii) reactivity with Ca^{2+}-activated PLA_2. The first observation provided proof of general LOOH character, while the second specified that the LOOH is PLOOH with an *sn*-2 peroxyl moiety. Other peroxides, besides the ChOOH and PCOOH described, remain to be identified and quantified. Peroxidized triacylglycerols might be significant products, since triacylglycerols comprise ~28% of total cellular lipid (see Materials and Methods section). Unlike cholesterol and PC, which exist mainly in cell membranes, triacylglycerols are localized in cytosolic lipid vacuoles. Formation of TGOOH would imply that intermediates such as $^1O_2$ generated by MC540 photoactivation in the plasma membrane (26,32) are long-lived enough to cause significant oxidation of cytosolic targets. Some of the unassigned peaks with short retention times (<10 min) could conceivably represent TGOOH (Figs. 2 and 3).

With HPLC–EC, we were able to detect significant levels of LOOH, including ChOOH and PCOOH, at the early stages of dye-sensitized photokilling. These levels were exceedingly low relative to overall lipid. For example, at a fluence of 0.045 J/cm^2, we estimate that the amount of ChOOH plus 18–22-min PCOOH produced was 60–65 pmol/10^8 cells, which translates to ~0.003 mol% of total lipid. These cells appeared to be completely viable by MTT and trypan blue exclusion assays, and >90% viable by clonal assay (Table 1), indicating that LOOH formation is an early event in relation to photokilling. Whether lipid peroxidation is causally linked to cell killing is not clear, but these observations, along with others described recently (18,40), are at least consistent with this possibility. Because damage to other targets may occur coincidentally with lipid peroxidation, the exact role of the latter is difficult to establish. For example, peroxidation-mediated lethality could result from (i) direct membrane damage and lysis, (ii) changes in membrane environments that alter critical protein functions such as ion pumping, or (iii) depletion of vital metabolites such as GSH, NAD(P)H, or ATP as a result of LOOH detoxification/repair.

MC540, the photosensitizing dye used in this study, is under intensive preclinical evaluation as an antitumor agent (41). The dye's phototherapeutic potential is exemplified by the fact that it binds preferentially to neoplastic cells in autologous remission bone marrow grafts and inactivates them when photoexcited by visible light, λ_{max} for the action spectrum and absorption spectrum

being ~568 nm (42). The proximal site of MC540-sensitized photodamage is believed to be the plasma membrane, since the dye localizes principally in this membrane, interacting strongly with lipid constituents (41). Previous studies showed that isolated membranes (liposomes, erythrocyte ghosts) and leukemia cells (L1210, K562) undergo potentially lethal lipid peroxidation when irradiated in the presence of MC540 (18,26,32). Peroxidation was assessed using relatively low-performance methods such as iodometric assay for "bulk" LOOH determination and TLC for preliminary LOOH identification. HPLC–EC represents a major advance in our ability to analyze peroxidized lipids in biological systems and to assess their cytotoxic potential. Further refinements of this approach, e.g., improved product resolution and characterization should enhance our ability to evaluate peroxide cytotoxicity, not only that associated with phototodynamic stress, but other types of oxidative stress as well.

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Effect of Dietary Carnosine on Plasma and Tissue Antioxidant Concentrations and on Lipid Oxidation in Rat Skeletal Muscle

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The effect of dietary carnosine supplementation on plasma and tissue carnosine and α-tocopherol concentrations and on the formation of thiobarbituric acid reactive substances (TBARS) in rat skeletal muscle homo-genates was evaluated. Plasma, heart, liver and hind leg muscle was obtained from rats fed basal semipurified diets or basal diets containing carnosine (0.0875%), α-tocopheryl acetate (50 ppm), or carnosine (0.0875%) plus α-tocopheryl acetate (50 ppm). Dietary carnosine supplementation did not increase carnosine concentrations in heart, liver and skeletal muscle. Dietary supplementation with both carnosine and α-tocopherol increased carnosine concentrations in liver 1.56-, 1.51- and 1.51-fold as compared with diets lacking carnosine, α-tocopherol or both carnosine and α-tocopherol, respectively. Dietary supplementation with both carnosine and α-tocopherol also increased α-tocopherol concentrations in heart and liver 1.38-fold and 1.68-fold, respectively, as compared to supplementation with α-tocopherol alone. Dietary supplementation with carnosine, α-tocopherol or both carnosine and α-tocopherol was effective in decreasing the formation of TBARS in rat skeletal muscle homogenate, with dietary α-tocopherol and α-tocopherol plus carnosine being more effective than dietary carnosine alone. The data suggest that dietary supplementation with carnosine and α-tocopherol modulates some tissue carnosine and α-tocopherol concentrations and the formation of TBARS in rat skeletal muscle homogenates.
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Carnosine (β-alanyl-L-histidine) is an endogenously synthesized dipeptide present in brain (1,2), cardiac muscle, kidney, stomach (1), olfactory bulbs (3) and skeletal muscle, with concentrations in skeletal muscle ranging from 1 to 20 mM (4). Carnosine is synthesized from β-alanine and histidine by carnosine synthetase, which has been found in brain, heart, liver (5), skeletal muscle (6) and olfactory bulb (7). Tissue carnosine concentrations can be influenced by diet. Dietary histidine deficiency reduces skeletal muscle carnosine concentrations in rats (8,9) while high dietary histidine supplementation (5%) has been shown to increase rat skeletal muscle carnosine concentrations (9). Supplementation with high concentrations (1.8–5.0%) of dietary carnosine also increases skeletal carnosine concentrations (9).

The biological function of carnosine is not fully understood, although carnosine has been suggested to act as both an antioxidant and a buffering agent (10). Carnosine has been shown to inhibit lipid oxidation catalyzed by iron (11), hydrogen peroxide-activated hemoglobin (11), singlet oxygen (12,13), lipoxygenase (11), peroxyl radicals (14,15) and hydroxyl radicals (16,17). The antioxidant activity of carnosine has also been demonstrated in oxidation model systems containing phosphatidylcholine liposomes (11), linoleic acid emulsions (14), skeletal muscle microsomes and sarcoplasmic reticulum (18,19). Due to its water solubility, carnosine would provide cells with an antioxidant system that could function in the cytosolic environment where water-soluble oxidation mediators, such as transition metals and oxygen radicals, can be present in high concentrations.

α-Tocopherol is a phenolic lipid-soluble antioxidant present in serum and in the membranes of tissues including heart, liver and skeletal muscle (20). Dietary supplementation of α-tocopherol can increase α-tocopherol concentrations in liver, serum, kidney, heart and skeletal muscle (21–24). An increase in tissue α-tocopherol concentrations has been shown to decrease susceptibility to lipid oxidation reactions, for example, in liver, kidney and skeletal muscle (25,26). α-Tocopherol deficiency in rats has been shown to be associated with a decrease in skeletal muscle carnosine concentrations suggesting that an interrelationship exists *in vivo* between α-tocopherol and carnosine levels (27).

Antioxidants have been postulated to retard various disease conditions including breast cancer, atherosclerosis and aging (28,29). An increase in the levels of endogenous antioxidants, such as α-tocopherol and carnosine through dietary means, may therefore increase the resistance of tissues against oxidation and retard the development of oxidation-related disease conditions. The objective of the present study was to determine how dietary carnosine, at a concentration equivalent to that in a diet containing 20% beef, would affect carnosine, anserine, histidine and α-tocopherol concentrations in both α-tocopherol-deficient and α-tocopherol-adequate rats. The effect of dietary carnosine and α-tocopherol on the formation of thiobarbituric acid reactive substances (TBARS) in rat skeletal muscle homogenates was also determined.

MATERIALS AND METHODS

Chemicals. Hexane and high-performance liquid chromatography (HPLC)-grade methanol were purchased from Curtin Matheson Scientific, Inc. (Houston, TX). Fluoaldehyde *o*-phthalaldehyde (OPA) reagent was from Pierce (Rockford, IL). Carnosine, α-tocopherol, α-tocopheryl acetate and sodium acetate were from Sigma (St. Louis, MO). All other chemicals were reagent-grade or purer.

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Abbreviations: HPLC, high-performance liquid chromatography; OPA, *o*-phthalaldehyde reagent; TBARS, thiobarbituric acid reactive substances.

Feeding regimen. Basal semipurified diets fed to rats were prepared as described in Table 1. A 2 × 2 factorial design was used. Groups included: (i) α-tocopheryl acetate (0 ppm) diet without added carnosine (–E–C); (ii) α-tocopheryl acetate (0 ppm) diet with 0.0875% carnosine (–E+C); (iii) α-tocopheryl acetate (50 ppm) diet without added carnosine (+E–C); and (iv) α-tocopheryl acetate (50 ppm) diet with 0.0875% carnosine (+E+C). The carnosine concentration used (0.0875%) in this study was chosen to be equivalent to that of a diet containing about 20% beef. Diets contained 10% stripped corn oil by weight and recommended levels of all other nutrients (including selenium) with the exception of α-tocopherol and carnosine. Diets were prepared monthly and stored at –20°C in sealed plastic bags. Weekly aliquots were stored at 4°C, and the remainder was disposed at week's end. Food was changed daily and remaining food was discarded.

Each group contained 30 animals at the beginning of the experiment. Tissues from eight randomly selected animals from each of the four treatment groups were analyzed. The animals were also the subjects of a mammary cancer study of six months' duration. Rats were treated with 65 mg/kg body weight of 7,12-dimethylbenz[a]anthracene by gavage tube at 55 d of age. Animals were placed on the experimental diets 10 d after gavage treatment for a total of 6 mon. All animals were fed the +E–C diet for a total of 3 wk before switching over to the experimental diets. Food and water were provided *ad libitum.* Mean body weights were not significantly different throughout the experiment (data not shown). Food intake was not measured nor were groups pair-fed as there were no significant differences in body weight.

Rats were exsanguinated by cardiac puncture using a heparinized syringe following metaphane anesthesia. Tissue from heart, liver and leg skeletal muscle, and blood plasma, were frozen immediately after collection and kept at –80°C until use.

Carnosine, anserine and histidine analysis. Heart (0.7 g), liver (2.5 g) and leg skeletal muscle (1.4 g) from six different animals of each dietary group were thawed at 4°C, minced, and homogenized in 3, 10 and 20 mL of 0.36 M perchloric acid, respectively, according to Kasziba *et al.* (1) using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY) at a setting of 4 for 2 min.

TABLE 1

Basal Diet Composition

Ingredient	g/kg Diet
Casein, vitamin-free	200
d,1-Methionine	3
Choline bitartrate	2
AIN-76A vitamin mix[a]	10
AIN-76 mineral mix	35
Cellulose	40
Corn starch	275
Glucose monohydrate	335
Stripped corn oil	100
Vitamin E acetate	± 0.05
Carnosine	± 0.875

[a]Vitamin mix contains no vitamin E.

Homogenates were heated in a boiling water bath for 5 min and centrifuged at 2,000 × *g* for 10 min with a Centrific™ centrifuge (Fisher Scientific, PA). Blood plasma (0.5 mL) was mixed with an equal volume of perchloric acid (0.36 M) and centrifuged at 5,000 × *g* for 10 min with an Eppendorf 5415C microcentrifuge (Madison, WI).

The supernatants from the perchloric acid extractions of heart, liver, skeletal muscle and blood plasma were filtered through a 0.45-μm filter membrane using a syringe. Filtrate (50 μL) was derivatized with 200 μL of OPA reagent for 2 min (30). Carnosine, anserine and histidine concentrations in the derivatized extract (100 μL) were determined by HPLC.

A 5-μm Hypersil ODS high-resolution end-capped column (250 mm × 46 mm) from Alltech Associated Inc. (Deerfield, IL) was utilized for HPLC analysis. The HPLC conditions described by Teahon and Rideout (30) were used. The column was initially equilibrated with 90% Solvent A [10% (vol/vol) methanol in 300 mM sodium acetate adjusted to pH 5.5 with glacial acetic acid] and 10% Solvent B [80% (vol/vol) methanol in 300 mM sodium acetate adjusted to pH 5.5 with glacial acetic acid]. The concentrations of Solvent B were gradually increased during the 25-min run (0 to 5 min, 10 to 20% Solvent B; 5 to 25 min, 20 to 30% Solvent B). The flow rate was 1.5 mL/min and the column temperature was maintained at 30°C. Carnosine, anserine and histidine were quantitated, using an FS 970 Spectrofluoro Monitor (Kratos Analytical Instruments, Ramsey, NJ) at 310 nm for excitation and 375 nm for emission, based on peak areas and standard curves obtained on 10–100 μg/mL of carnosine and histidine and 100–500 μg/mL anserine.

α-Tocopherol analysis. Heart (0.6 g), liver (0.9 g) and leg skeletal muscle (0.6 g) from six different animals of each dietary group were thawed at 4°C, minced and homogenized with 4 mL ethanol (31) using a Polytron homogenizer at a setting 4 for 2 min. Blood plasma (0.5 mL) was mixed with an equal volume of ethanol. The resulting homogenates (1 mL) were mixed with 3.5 mL of hexane and centrifuged at 2,000 × *g* for 10 min. The hexane extract (3 mL) was dried under nitrogen followed by reconstitution in either 0.4 mL (for –E–C and –E+C group samples) or 2.5 mL (for +E–C and +E+C group samples) of hexane before HPLC analysis.

The analytical column used for α-tocopherol measurements was a 5-μm Econosil ODS column (250 mm × 46 mm) from Alltech Associated Inc. Samples (50 μL) were injected into the column using 100% HPLC-grade methanol as the eluting solvent at a flow rate of 1.5 mL/min. α-Tocopherol was detected and quantified at 294 nm with a Waters 490 Multiwavelength Detector (Milford, MA) (32) based on a standard curve obtained on 1–5 μg/mL α-tocopherol standard samples.

Oxidation of skeletal muscle homogenates. Hind-leg skeletal muscles (14 g) from eight different animals from each dietary group were homogenized separately in 100 mL of precooled phosphate buffer (0.1 M, pH 7.4) with a Waring blender for 2 min at 4°C. The homogenates were filtered through three layers of cheesecloth to remove connective tissues, and the filtrates were incubated in a shaking water bath at 37°C for 10 h. The extent of lipid oxida-

tion of the muscle homogenate (3 mL) was determined at various time points by measuring TBARS as described by Sinnhuber and Yu (33). TBARS were expressed as μg malondialdehyde/g muscle. Simultaneously, 0.9 mL of muscle homogenate was mixed with 0.1 mL of butylated hydroxytoluene (BHT; 0.2%) and kept at –80°C until α-tocopherol was measured. Muscle homogenates were mixed with ethanol (1 mL), and α-tocopherol was extracted with hexane and quantitated by HPLC as described above.

Statistical analysis. Carnosine, anserine, histidine and α-tocopherol analyses were done on tissue samples from six different animals on each dietary treatment. Oxidation studies were carried out on muscle from eight different animals on each dietary treatment. TBARS and α-tocopherol concentrations within an oxidation period were compared by analysis of variance utilizing a randomized complete block design to partition the effects of daily variations (34). Statistical analyses were done using the Statistical Analysis System (SAS Institute, Inc., Cary, NC) using the Duncan's multiple range test to determine the significance of difference ($P \leq 0.05$) between mean values.

RESULTS

Carnosine, anserine, histidine and α-tocopherol levels in rat tissues. Carnosine was measurable in heart, liver and skeletal muscle but not in blood plasma (Table 2). Tissue carnosine concentrations were in the order of skeletal muscle > heart > liver in all animals tested. Carnosine concentrations in heart and skeletal muscle were not significantly affected by the dietary treatments (Table 2). Dietary supplementation with carnosine (–E+C) or α-tocopherol (+E–C) did not affect carnosine concentrations in liver; however, supplementation with +E+C increased carnosine concentrations in liver 1.56-, 1.51- and 1.51-fold when compared with the –E–C, –E+C and +E–C diets, respectively (Table 2).

Anserine was detected in skeletal muscle but not in heart, liver or blood plasma (Table 3). None of the dietary treatments significantly affected anserine concentrations in skeletal muscle (Table 3). Histidine was detected in heart, liver, skeletal muscle and plasma with tissue concentrations in the order of heart > liver > skeletal muscle in all animals tested (Table 4). Histidine concentrations in liver and plasma were not significantly affected by the dietary treatments (Table 4). Histidine concentrations in heart were increased 1.46-fold by dietary supplementation with carnosine (–E+C) when compared with tissue values for the unsupplemented diet group (–E–C; Table 4). However, dietary supplementation with tocopherol (+E–C) or both carnosine and α-tocopherol (+E+C) did not affect histidine concentrations in heart (Table 4). Histidine concentrations in skeletal muscle were unaffected by dietary supplementation with carnosine (–E+C) or α-tocopherol (+E–C) when compared with tissue values for unsupplemented rats (–E–C; Table 4). Dietary supplementation with both carnosine and α-tocopherol (+E+C) increased histidine concentrations in skeletal muscle 1.18-, 1.29- and 1.09-fold when compared with the values for animals on the –E–C, –E+C and +E–C diets, respectively (Table 4).

α-Tocopherol was detected in all samples tested, with tissue concentrations in the order of heart > liver > skeletal muscle (Table 5). α-Tocopherol concentrations in heart, liver, skeletal muscle and plasma from rats fed the α-tocopherol-supplemented diet (+E–C) were increased 28.6-, 55.1-, 24.1- and 17.8-fold, respectively, as compared with tissues or plasma from rats on unsupplemented diets (–E–C; Table 5). α-Tocopherol concentrations in heart and liver were increased 1.38- and 1.68-fold, respectively, by carnosine/α-tocopherol supplementation (+E+C) when compared to the values for rats on the +E–C diet. Carnosine/α-tocopherol supplementation did not significantly increase α-tocopherol concentrations in skeletal muscle and serum when compared with the respective +E–C values (Table 5). α-Tocopherol concentrations in heart, liver, skeletal muscle and plasma from rats fed the α-tocopherol-deficient diet (–E–C) were not affected by carnosine supplementation (–E+C; Table 5). The effect of vitamin E deficiency was histologically assessed by Dr. C.B. Hong of the University of Kentucky Livestock Disease Diagnostic Center (Lexington, KY). Examination of liver, skeletal muscle and intestinal sections of vitamin E-adequate (+E–C) and vitamin E-deficient (–E–C) rats revealed no lesions associated with vitamin E or selenium deficiency.

TABLE 2

Carnosine Concentrations (mg carnosine/100 g tissue or plasma, mean ± standard errors, n = 6) in Tissues and Plasma of Rats Fed Different Diets[a]

	–E–C[b]	–E+C[c]	+E–C[d]	+E+C[e]
Heart	18.92 ± 0.35^x	19.49 ± 0.45^x	16.10 ± 1.79^x	18.64 ± 2.44^x
Liver	4.31 ± 0.21^x	4.46 ± 0.15^x	4.46 ± 0.44^x	6.74 ± 0.37^y
Muscle	87.41 ± 8.81^x	87.84 ± 6.9^x	84.65 ± 6.9^x	84.0 ± 6.78^x
Plasma	n.d.	n.d.	n.d.	n.d.

[a]Numbers with different letters (x,y) within the same row are significantly different ($P \leq 0.05$); n.d., not detectable.

[b]Rats fed diet without tocopherol and carnosine supplementation.

[c]Rats fed diet supplemented with 0.0875% carnosine.

[d]Rats fed diet supplemented with 50 ppm α-tocopheryl acetate.

[e]Rats fed diet supplemented with 0.0875% carnosine and 50 ppm α-tocopheryl acetate.

TABLE 3

Anserine Concentrations (mg anserine/100 g tissue or plasma, mean ± standard errors, n = 6) in Tissues and Plasma of Rats Fed Different Diets[a]

	–E–C[b]	–E+C[c]	+E–C[d]	+C+C[e]
Heart	n.d.	n.d.	n.d.	n.d.
Liver	n.d.	n.d.	n.d.	n.d.
Muscle	500.8 ± 32.7[x]	461.8 ± 24.1[x]	519.7 ± 12.6[x]	468.4 ± 21.1[x]
Plasma (μg/g)	n.d.	n.d.	n.d.	n.d.

[a]Numbers with superscript x are not significantly different ($P \geq 0.05$). n.d., not detectable.

[b]Rats fed diet without tocopherol and carnosine supplementation.

[c]Rats fed diet supplemented with 0.0875% carnosine.

[d]Rats fed diet supplemented with 50 ppm α-tocopheryl acetate.

[e]Rats fed diet supplemented with 0.0875% carnosine and 50 ppm α-tocopheryl acetate.

TABLE 4

Histidine Concentrations (mg histidine/100 g tissue or plasma, mean ± standard errors, n = 6) in Tissues and Plasma of Rats Fed Different Diets[a]

	–E–C[b]	–E+C[c]	+E–C[d]	+E+C[e]
Heart	31.38 ± 2.46[x]	45.79 ± 2.31[y]	31.70 ± 3.33[x]	36.55 ± 2.91[x,y]
Liver	19.10 ± 0.46[x]	20.64 ± 0.55[x]	19.42 ± 1.12[x]	20.55 ± 0.33[x]
Muscle	11.82 ± 0.46[x,y]	10.82 ± 0.46[x]	12.89 ± 0.59[y,z]	14.00 ± 0.70[z]
Plasma (μg/mL)	21.88 ± 2.32[x]	22.66 ± 1.69[x]	24.50 ± 1.86[x]	24.81 ± 0.58[x]

[a]Numbers with different letters (x,y,z) within the same row are significantly different ($P \leq 0.05$).

[b]Rats fed diet without tocopherol and carnosine supplementation.

[c]Rats fed diet supplemented with 0.0875% carnosine.

[d]Rats fed diet supplemented with 50 ppm α-tocopheryl acetate

[e]Rats fed diet supplemented with 0.0875% carnosine and 50 ppm α-tocopheryl acetate.

TABLE 5

Tocopherol Concentrations (μg α-tocopherol/g muscle, mean ± standard deviation) in Tissues of Rats Fed Different Diets[a]

	–E–C[b]	–E+C[c]	+E–C[d]	+E+C[e]
Heart	1.86 ± 0.27[x]	2.85 ± 0.20[x]	53.15 ± 1.80[y]	73.33 ± 7.06[z]
Liver	1.15 ± 0.08[x]	1.93 ± 0.20[x]	63.40 ± 7.22[y]	106.5 ± 13.76[z]
Muscle	1.04 ± 0.10[x]	1.83 ± 0.14[x]	25.05 ± 3.03[y]	27.63 ± 2.93[y]
Plasma	0.6 ± 0.04[x]	0.5 ± 0.04[x]	10.7 ± 0.29[y]	9.6 ± 0.16[y]

[a]Numbers with different letters (x,y,z) within the same row are significantly different ($P \leq 0.05$).

[b]Rats fed diet without tocopherol and carnosine supplementation.

[c]Rats fed diet supplemented with 0.0875% carnosine.

[d]Rats fed diet supplemented with 50 ppm α-tocopheryl acetate.

[e]Rats fed diet supplemented with 0.0875% carnosine and 50 ppm α-tocopheryl acetate.

TBARS and α-tocopherol concentrations in incubated skeletal muscle homogenates. Skeletal muscle homogenates obtained from rats fed diets lacking both carnosine and α-tocopherol (–E–C) were the least oxidatively stable ($P \leq 0.05$) of all muscle samples tested with TBARS increasing 8.7-fold between 0 and 10 h of incubation (Fig. 1). Dietary carnosine supplementation (–E+C) significantly ($P \leq 0.05$) decreased the rate of TBARS formation by 14.5 to 27.5% during the oxidation period (0 to 10 h) as compared with TBARS of unsupplemented muscle homogenate (–E–C; Fig. 1). Dietary α-tocopherol supplementation (+E–C) significantly ($P \leq 0.05$) decreased the rate of TBARS formation during the entire incubation when compared with unsupplemented homogenate (–E–C; Fig. 1). Muscle homogenate from rats supplemented with both carnosine and α-tocopherol (+E+C) showed 90.5, 88.8 and 30.1% less TBARS after 6 h of oxidation compared to –E–C, –E+C and +E–C, respectively (Fig. 1). TBARS in

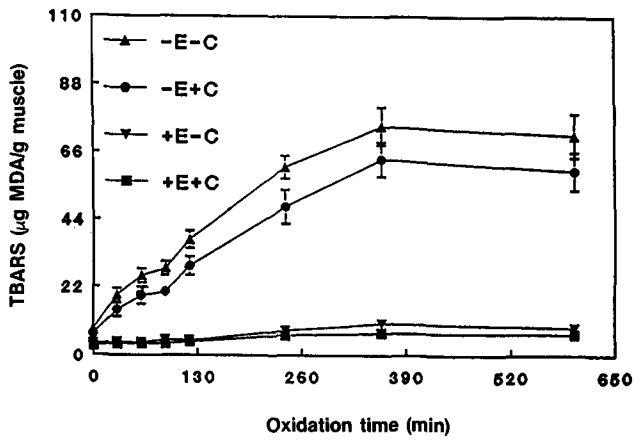

FIG. 1. Thiobarbituric acid reactive substances (TBARS) formation in muscle homogenates from rats fed diets not supplemented with carnosine and α-tocopherol (–E–C), supplemented with carnosine (0.0875%; –E+C), α-tocopherol (50 ppm; +E–C), or both carnosine (0.0875%) and α-tocopherol (50 ppm; +E+C) incubated for the indicated periods of time. Data points and error bars represent means (n = 8) and standard errors. Some standard errors lie within the symbols. MDA, malondialdehyde.

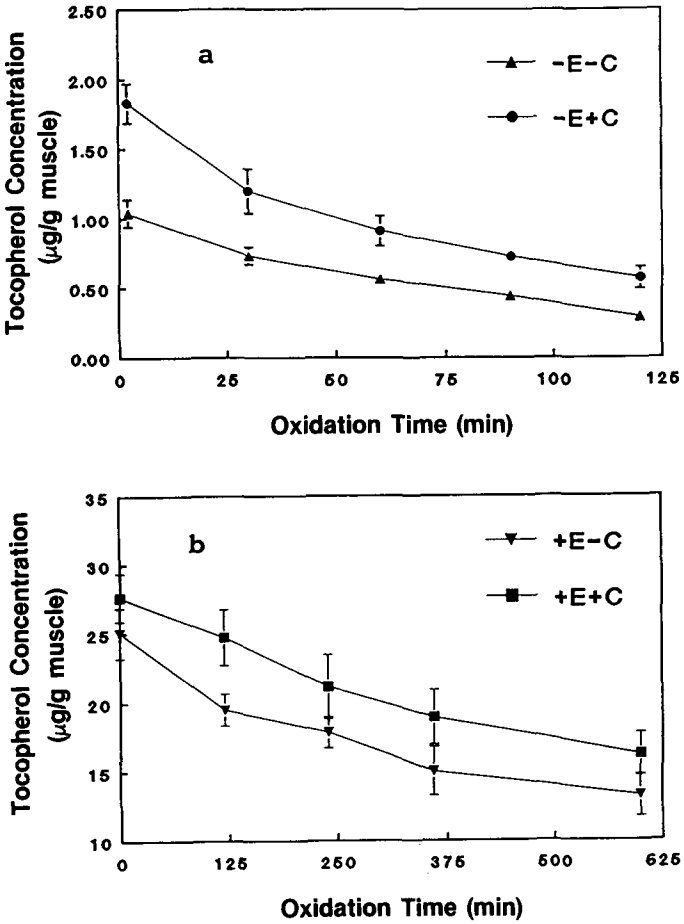

FIG. 2. α-Tocopherol concentrations of muscle homogenates from rat fed diets (a) not supplemented with carnosine and α-tocopherol (–E–C) and supplemented with carnosine (0.0875%; –E+C); (b) supplemented with α-tocopherol (50 ppm; +E+C); incubations were for the indicated periods of time. Data points and error bars represent means (n = 8) and standard errors. Some standard errors lie within the symbols.

muscle homogenates obtained from rats fed the +E+C diets were significantly ($P \leq 0.05$) lower than TBARS in homogenates from +E–C rats at all time points tested.

Muscle homogenates from α-tocopherol-supplemented rats (+E–C and +E+C) had higher α-tocopherol concentrations ($P \leq 0.05$) than homogenates without α-tocopherol supplementation (–E–C and –E+C) for all incubation periods (Fig. 2). Carnosine supplementation resulted in homogenates from rats fed diets with ($P \leq 0.05$; Fig. 2b) or without ($P \leq 0.05$; Fig. 2a) α-tocopherol. α-Tocopherol concentrations decreased over the course of the incubation period. Similar disappearance rates were observed for the –E–C and –E+C homogenates (Fig. 2a) as well as for the +E–C and +E+C homogenates (Fig. 2b).

DISCUSSION

Carnosine and α-tocopherol are antioxidants whose concentrations may be affected by diet (9,22,23). The present studies show that supplementation with 0.0875% carnosine did not increase carnosine concentrations in heart, liver and skeletal muscle (Table 2). Carnosine was not detected in plasma (Table 2), which may be due to the presence of carnosinase that hydrolyzes carnosine into β-Ala and histidine (35). However, plasma histidine concentrations were not elevated in carnosine-supplemented rats (Table 4). It is possible that diet-derived carnosine or the histidine derived from hydrolysis of plasma carnosine may be rapidly removed from plasma. Heart histidine concentrations were observed to increase in rats fed –E+C diets, while skeletal muscle histidine concentrations were observed to increase in rats fed the vitamin E-containing diets (+E–C and +E+C). How these diets were affecting heart and muscle histidine concentrations is unknown. Kasziba *et al.* (1) reported that anserine was present in heart and skeletal muscle of rats; however, in the present study, anserine was only detected in skeletal muscle (Table 3).

Tamaki *et al.* (9) reported that 1.8% dietary carnosine supplementation increased endogenous carnosine concentrations of skeletal muscle of rats fed a histidine-free diet. The absence of changes in carnosine concentrations in skeletal muscle seen in the present study could be due to the low, albeit physiologically relevant, level of carnosine supplementation used (0.0875%). Nevertheless, carnosine concentrations were observed to increase in liver when rats were fed diets containing both α-tocopherol and carnosine. It is not clear why supplemented carnosine was preferentially transported and stored in liver.

Dietary α-tocopherol supplementation increased α-tocopherol concentrations in heart, liver, skeletal muscle and plasma (Table 4) as has been reported by other investigators (21–23). α-Tocopherol concentrations were further increased by carnosine supplementation (+E+C) in heart, liver, skeletal muscle and plasma, although the observed increases in skeletal muscle and plasma were not

significant ($P \geq 0.05$). McManus (27) found that vitamin E deficiency causes over a 60% decrease in skeletal muscle carnosine concentrations in rabbits. Elevated α-tocoperhol concentrations due to carnosine supplementation and decreased carnosine concentrations due to Vitamin E deficiency suggest that an interrelationship between α-tocopherol and carnosine exists *in vivo.*

Diets containing either carnosine (–E+C), α-tocopherol (+E–C) or both carnosine and α-tocopherol (+E+C) were effective in inhibiting lipid oxidation in skeletal muscle when compared to values for the unsupplemented diet (–E–C; Fig. 1). Carnosine concentrations of skeletal muscle from all dietary treatments were similar (Table 2). However, α-tocopherol concentrations of skeletal muscle homogenate from carnosine-supplemented rats were slightly higher than from unsupplemented rats. Therefore, the lower rate of TBARS formation in muscle homogenate from –E+C, +E–C +E+C could be due to higher α-tocopherol concentrations (Fig. 2). α-Tocopherol in –E–C and –E+C (Fig. 2a) as well as in +E–C and +E+C (Fig. 2b) homogenates disappeared in the muscle homogenate at similar rates, suggesting that dietary carnosine supplementation did not increase the stability of α-tocopherol. Although an *in vivo* interrelationship between α-tocopherol and carnosine might exist, carnosine is not thought to be capable of regenerating oxidized α-tocopherol (11,36). Therefore, any increases in antioxidant activity and/or sparing of α-tocopherol by carnosine could be due to differences in the ability of these antioxidants to inactivate lipid- and water-soluble oxidation catalysts.

In conclusion, dietary carnosine supplementation did not affect carnosine concentrations in heart, liver and skeletal muscle. Dietary supplementation with both carnosine and α-tocopherol increased carnosine concentration in liver and α-tocopherol concentrations in liver and heart as compared with diets supplemented with α-tocopherol alone. Dietary supplementation with carnosine, α-tocopherol, or both carnosine and α-tocopherol effectively decreased lipid oxidation in rat skeletal muscle.

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Effects of Sodium Butyrate on the Transfer of Arachidonic Acid to Phosphatidylcholine in a Clonal Oligodendrocyte Cell Line (CB-II)

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The effect of sodium butyrate on membrane phospholipid metabolism in a neonate rat cerebellum derived clonal oligodendrocyte cell line (CB-II) was investigated. Sodium butyrate is an agent known to induce cell differentiation and morphological transformations. A comparison of the *in vivo* phospholipid labeling patterns obtained by incubating CB-II cells with [^{3}H]choline, [^{14}C]myristic acid or [^{3}H]arachidonic acid indicated that butyrate altered the route of acylation–deacylation in phosphatidylcholine (PC) biosynthesis. Using an *in vitro* incubation system containing homogenates of CB-II cells, the largest proportion of radioactivity was found in PC, and addition of sodium butyrate resulted in a further increase in the transfer of arachidonic acid to PC, but not to phosphatidylinositol. Similar results were obtained when this *in vitro* acylation activity was tested using homogenates from sodium butyrate pretreated cells. The butyrate effect was observed regardless of whether or not exogenous lysophosphatidylcholine (LPC) was added to the incubation system. Addition of butyrate did not result in a change in the activity of LPC:acyl-CoA (coenzyme A) acyltransferase (EC 2.3.1.23) in CB-II cells upon incubating cell homogenates with [1-^{14}C]arachidonoyl-CoA and LPC. However, when cell homogenates were incubated with [^{3}H]arachidonic acid in the presence of 2.5–10 mM sodium butyrate, arachidonoyl-CoA synthesis was stimulated. A time course study demonstrated that significant stimulation occurred after three minutes. Taken together, the results suggest that in CB-II cells, sodium butyrate stimulates the transfer of arachidonic acid into PC and that this effect is at least partially due to a stimulation of arachidonoyl-CoA ligase (EC 6.2.1.3).
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Sodium butyrate is known to stimulate growth and differentiation in a number of cell culture systems (1). For example, the action of butyrate was shown to be associated with the extension of neurite-like processes in a neuroblastoma cell line (2). In C6 glioma cells, the effect of butyrate was related to the ability of butyrate to modulate hormone-mediated induction of glial fibrillary acidic protein and S-100 (3). Butyrate was also shown to induce nerve growth factor gene expression in primary brain monolayer cultures (4) and the expression of *fos* (5,6), *sis* proto-oncogenes, as well as genes of the extracellular matrix protein in F-98 rat glioma cells (6). It is likely that the morphological changes induced by butyrate are associated with changes in membrane lipid compoisition (7,8). Our recent study with a neonate cerebellum-derived clonal oligodendrocyte cell line (CB-II) has correlated the morphological transformation induced by butyrate with changes in membrane phospholipids profiles (9).

It has been well documented that arachidonic acid metabolism in the cell is dependent on the deacylation–reacylation cycle mediated by phospholipase A_2 and lysophopholipid:acyl-CoA (coenzyme A) acyltransferases (10). Reacylation with a fatty acid also depends on the ability of this fatty acid to be converted to acyl-CoA *via* acyl-CoA ligase. Various factors, including detergents and lipid intermediates, such as acyl-CoA and lysophospholipids (11), are known to alter the reacylation process (12,13). The increase in free arachidonic acid and prostanoid in the resident macrophage was shown to be linked to the inhibition of lysophospholipid acyltransferase (14). Phorbol esters and diacylglycerols could also inhibit the incorporation of arachidonate into phospholipids in human platelets through inactivation of both arachidonoyl-CoA ligase and arachidonoyl-CoA:lysophosphatide acyltransferase (15). In rat mesangial cells, stimulation by interleukin-1 of prostaglandin synthesis was related to an increase in both phospholipase A_2 and acyltransferase activities (16). We have previously shown that exposure of CB-II cells to butyrate resulted in an increase in the incorporation of labeled arachidonate into phosphatidylcholine (PC) and a decrease in labeling of phosphatidylethanolamine (PE) (9). However, it is not clear whether the lipid changes are associated with a change in *de novo* biosynthesis or in the acylation–deacylation pathway. In this study, we examined the mechanism underlying the action of sodium butyrate on membrane phospholipids of CB-II cells by incubating cells with lipid precursors and by assaying the arachidonate transfer system *in vitro*. Our results point at a specific effect of butyrate by enhancing the synthesis of arachidonoyl-CoA.

MATERIALS AND METHODS

Materials. Adenosine triphosphate (ATP), bovine serum albumin (BSA; essentially fatty acid free), CoA, bovine brain lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), 1-myristoyl lysophosphatidylcholine (LPC), palmitic acid, PC, phosphatidylinositol (PI), phosphatidylserine (PS), PE, and ethanolamine plasmalogen (PEpl) and sodium butyrate were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Arachidonic acid (886 mCi/mmol), [1-^{14}C]arachidonoyl-CoA (46.3 mCi/mmol), [^{3}H]arachidonic acid (240 Ci/mmol), [*methyl*-^{3}H]choline chloride (85.1 Ci/mmol) and [1-^{14}C]myristic acid (58 mCi/mmol) were purchased from NEN/DuPont (Wilmington, DE). DM-160 media were purchased from Kyokuto Pharmaco-

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Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; CB-II, neonate rat cerebellum-derived clonal oligodendrocyte; CoA, coenzyme A; FBS, fetal bovine serum; HPTLC, high-performance thin-layer chromatography; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PBG, glucose supplemented phosphate buffer; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEpl, ethanolamine plasmalogen; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triacylglycerol(s).

logical Industrial Co. (Tokyo, Japan); fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY) and Hyclone Laboratories Inc. (Logan, UT). Silica gel 60 (10 cm × 10 cm) high-performance thin-layer chromatography (HPTLC) plates and organic solvents were from E. Merck (Darmstadt, Germany). T-25 and T-75 plastic tissue culture flasks and 15- and 50-mL polypropylene centrifuge tubes were purchased from Corning Glass Inc. (Corning, NY). Disposable borosilicate glass culture tubes were from Kimble, a Division of Owen–Illinois (Toledo, OH).

Cell culture. The clonal oligodendrocytes (CB-II) derived from neonate rat cerebellum were cultured in DM 160 medium supplemented with 10% FBS and 50 μg of gentamicin/mL, as described previously (9). These cells had been characterized earlier as glial fibrillary acidic protein negative and galactocerebroside positive and, more recently, as cyclic nucleotide phosphohydrolase positive (data not shown). Cell passages 50–70 were used for the experiments, and cells were normally maintained in T-75 flasks (Corning Glass, Inc.). In a typical experiment, 1.5 to 2 × 10^6 or 2.5 × 10^5 cells were subcultured in T-75 or T-25 flasks, respectively, for three days.

[^{3}H]Arachidonic acid, [^{3}H]choline and [^{14}C]myristic acid incorporation into intact CB-II cells. The incorporation of lipid precursors into intact CB-II cells was followed by subculturing the cells in DM 160 medium supplemented with 10% FBS and 50 μg of gentamicin in T-25 flasks for at least two days. The culture medium was then changed to DM 160 supplemented with 2.5% FBS, in the absence or presence of 2.5 mM sodium butyrate. Labeled precursors, such as 0.5 μCi [^{3}H]arachidonic acid, 4 μCi [^{3}H]choline, or 0.5 μCi [^{14}C]myristic acid, were added and cells were cultured for another 24 h, as described previously (9). At the end of the labeling period, the culture medium was removed, the cells were rinsed once with 0.02 M phosphate buffer (pH 7.4) containing 0.1% glucose (PBG) to remove excess labeled precursors and the reaction was stopped by adding 1.3 mL of ice-cold methanol to the culture. The cells in methanol were transferred to a 13 × 100 mm borosilicate glass test tube, and then 2.7 mL of chloroform and 1 mL of water were added to extract lipids (9).

In vitro *assays of [^{3}H]arachidonic acid transfer.* In the *in vitro* assay system, cells were cultured in T-75 flasks in DM 160 medium supplemented with 10% FBS and 50 μg of gentamicin/mL for at least three days. Subconfluent cultures were removed by trypsinization and aliquots taken for cell counting. After washing with PBG, cells were suspended (2 × 10^6 per mL) in 50 mM Tris-HCl (pH 7.4) and were homogenized with a Potter-Elvehjem homogenizer. A small portion of the cell homogenate was removed for protein assay (17) using BSA as standard. In some experiments, cells were quickly frozen in liquid nitrogen and stored until use. Cells were homogenized with a Potter-Elvehjem homogenizer before the assay. Aliquots of 0.5 mL cell homogenate were incubated in a test tube with 1 μCi of [^{3}H]arachidonic acid, 0.1% fatty acid-free BSA, 0.1 mM CoA, 0.3 mM dithiothreitol, 2.5 mM ATP, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) in the absence or presence of sodium butyrate in a total volume of 1 mL according to the method of Lin *et al.* (18). All incubations were carried out at 37°C for 10 min unless otherwise indicated. In some experiments, 4 μM LPI, 4 μM LPS, 20 μM LPC or 20 μM bovine brain LPE were used according to MacQuarrie *et al.* (13). The lipids, cofactors and sodium butyrate, together with cell homogenates in buffer, were kept in an ice-cold water bath and vortexed vigorously for 20 s prior to incubation. After incubation, the reaction was stopped by adding four volumes of chloroform/methanol (2:1, vol/vol).

Assay of LPC:arachidonoyl-CoA acyltransferase activity. Homogenates from approximately 5 × 10^5 cells were incubated with 50 nCi [1-^{14}C]arachidonoyl-CoA and lysophospholipids in the absence or presence of sodium butyrate and 50 mM Tris-HCl (pH 7.4) in a total of 1 mL at 37°C for 20 min. The amounts of lysophospholipids used were 4 μM LPI, 4 μM LPS, 20 μM LPC or 20 μM bovine brain LPE according to MacQuarrie *et al.* (13). The bovine brain LPE contained 60% alkenyl groups and 40% acyl groups. After incubation, the reaction was stopped by adding four volumes of chloroform/methanol (2:1, vol/vol), and the lipids were extracted and separated by two-dimensional HPTLC (see next section). The radioactivities associated with phospholipids were measured in a Beckman (Palo Alto, CA) scintillation counter and are expressed in pmol/mg protein.

Lipid extraction and separation. For extraction of lipids, four volumes of chloroform/methanol (2:1, vol/vol) were added to the cell suspension. After mixing, the tubes were briefly centrifuged at 2000 rpm to allow phase separation. The lower organic phase was removed and transferred into test tubes. In order to obtain a complete recovery of the acidic phospholipids, the aqueous phase was further extracted by adding two volumes of chloroform/methanol/12N HCl (225:75:0.75, by vol). The acidic organic phase was then removed and neutralized with 4N NH_4OH (about 1 drop) before combining it with the first organic extract. The combined organic extracts were evaporated to dryness, redissolved in chloroform, and applied to HPTLC plates (Kieselgel 60, 10 × 10; E. Merck, Darmstadt, Germany). Phospholipids, namely PI, PS, PC, PEpl and PE, and free fatty acid were separated using a two-dimensional system according to Sun and Horrocks (19) with some modification (9). Briefly, first the plate was developed using chloroform/methanol/acetone/12M NH_4OH (35:20:5:5, by vol). Then the plate was removed from the tank, dried, exposed to HCl fumes for 3 min, and then residual HCl fumes were removed by a stream of warm air for 10 min. The plates were then developed in the second dimension with chloroform/methanol/acetone/glacial acetic acid/0.2 M aqueous ammonium acetate (35:15:14:1:1.75, by vol). After development, the lipid fractions were visualized by exposure to iodine vapor, and the individual phospholipid fractions were scraped into scintillation vials for radioactivity counting. In some experiments, the neutral lipids were removed after separation in the first dimension, extracted and further separated on another HPTLC plate using hexane/diethyl ether/glacial acetic acid (70:30:3.5, by vol). The lipid bands corresponding to triacylglycerols (TG) were used for radioactivity counting.

Assay of arachidonoyl-CoA ligase activity. The synthesis of arachidonoyl-CoA was followed according to the method by Morisaki *et al.* (20), with some modifications. Briefly, homogenates from 5×10^5 cells (approximately 100 μg protein) were incubated with 50 nCi [^{14}C]arachidonic acid, 0.1% fatty acid-free BSA, 0.1 mM CoA, 0.3 mM dithiothreitol, 2.5 mM ATP, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) in the presence and absence of sodium butyrate in a total volume of 250 μL. Incubations were carried out at 37°C for various time periods ranging from 20 s to 10 min. The reaction was terminated by adding 1 mL of Dole's mixture, i.e., isopropanol/heptane/2M H_2SO_4 (40:10:2, by vol) (21). The sample was mixed for 5 min, and then 0.35 mL of water and 0.5 mL of heptane were added. After further mixing, the upper phase was removed, and the lower phase was washed three times with 0.6 mL of heptane containing 5 mg/mL of nonradioactive palmitic acid as carrier (22). Subsequently, the lower phase was taken for radioactivity counting. Levels of arachidonoyl-CoA formed are expressed in nmol/mg protein.

RESULTS

Effect of sodium butyrate on the incorporation of labeled arachidonate, myristate and choline into phospholipids of CB-II cells. When CB-II cells were incubated in the growth medium supplemented with 2.5% FBS containing either [^{3}H]arachidonate or [^{14}C]myristate for 24 h, both labeled fatty acids were incorporated into the cell phospholipids, although each of the labeled fatty acid gave rise to a unique labeling pattern. As shown in Table 1, a greater proportion of labeled myristate was incorporated into PC, whereas labeled arachidonate was incorporated into PC as well as PE and PEpl. With cells labeled with [^{3}H]arachidonate, there was a 25% increase in labeling of PC and a 40% decrease in labeling of PE upon exposure to butyrate (2.5 mM) for 48 h (Table 1). However, this effect of butyrate on PC synthesis was not observed when cells were incubated with [^{14}C]myristic acid (Table 1). In a subsequent experiment, PC biosynthesis in CB-II cells was further probed by incubating cells with [^{3}H]choline. Over 90% of the radioactivity from [^{3}H]choline was found in PC (Table 2). Exposure of cells to butyrate (2.5 mM) for 24 h resulted in a decrease in labeled LPC and sphingomyelin and a small increase in labeled PC.

Effects of sodium butyrate on the incorporation of [^{3}H]arachidonic acid into membrane phospholipids in an in vitro *incubation system.* In this series of experiments, an *in vitro* incubation system was used to assess the incorporation of labeled arachidonate into membrane phospholipids. In the absence of lysophospholipids, [^{3}H]arachidonate was preferentially incorporated into PC of the cell homogenates, but smaller amounts were also found in PI and PE (Fig. 1). Addition of butyrate to the incubation system containing the cell homogenates led to a dose-dependent increase in labeling of PC, but labeling of PI and PE was not altered (Table 3). When exogenous LPC (20 μM) and LPI (4 μM) were added to the *in vitro* incubation system, there was an increase in incorporation of [^{3}H]arachidonate into PC (37%) and PI (80%), respectively (Fig. 2). Nevertheless, further addition of butyrate to systems containing LPC and LPI only enhanced the labeling of PC, but not that of PI (Fig. 2). In a similar experiment, sodium butyrate also enhanced the transfer of arachidonic acid

TABLE 1

***In Vivo* Effect of Sodium Butyrate on Fatty Acid Incorporation into Phospholipids in Intact CB-II Cells[a,b,c]**

	[^{3}H]Arachidonate sodium butyrate		[^{14}C]Myristate sodium butyrate	
Phospholipid	–	+	–	+
	(% of total)			
PI	10.4 ± 0.83	11.4 ± 1.55	6.42 ± 0.80	6.29 ± 0.68
PS	3.29 ± 0.15	3.03 ± 0.31	4.18 ± 0.21	3.58 ± 0.22
PC	37.1 ± 3.48	46.3 ± 2.32[d]	78.4 ± 1.30	79.9 ± 1.87
PEpl	23.6 ± 1.97	24.5 ± 1.97	2.19 ± 0.21	3.42 ± 1.01
PE	24.0 ± 2.08	13.7 ± 2.08[d]	8.79 ± 0.45	6.68 ± 0.36[d]
Total dpm	82801 ± 3542	75125 ± 2760	45883 ± 2632	40979 ± 4531

[a]Radioactivity of the lipids disintegration per minute (dpm) is expressed as % of total (mean ± SD) from three determinations. CB-II, neonate rat cerebellum-derived clonal oligodendrocyte.

[b]CB-II cells (2.5×10^5) were subcultured in DM 160 medium supplemented with 10% fetal bovine serum (FBS) medium for three days. The medium was changed to DM 160 medium supplemented with 2.5% FBS in the absence (–) or presence (+) of 2.5 mM sodium butyrate and incubated together with 0.5 μCi of the indicated fatty acid for 24 h.

[c]Procedures for separation and measurement of the radioactivities of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), plasmalogen ethanolamine (PEpl) and phosphatidylethanolamine (PE) are described in the Materials and Methods section.

[d]Denotes values are significantly different comparing butyrate treatment with control ($P \leq 0.05$) based on nonpaired Student's *t*-test.

TABLE 2

***In Vivo* Effect of Sodium Butyrate on [^{3}H]Choline Incorporation into Phospholipids of Intact CB-II Cells[a,b,c]**

Phospholipid	Sodium butyrate	
	–	+
	(% of total)	
LPC	1.54 ± 0.16	1.07 ± 0.15[d]
Sph	8.52 ± 0.18	7.05 ± 0.36[d]
PC	90.0 ± 0.28	91.3 ± 0.42
Total dpm	121503 ± 14593	113201 ±

[a]Radioactivity of the lipids (dpm) is expressed as % of total (mean ± SD) from three determinations. Abbreviations as in Table 1.
[b]CB-II cells (2.5×10^5) were subcultured in DM 160 supplemented with 10% FBS medium, which was later changed to DM 160 supplemented with 2.5% FBS containing 4 μCi [^{3}H]choline in the absence (–) or presence (+) of 2.5 mM sodium butyrate and incubated for 24 h.
[c]The procedures for separation and measurement of the radioactivities of lysophosphatidylcholine (LPC), sphingomyelin (Sph) and PC are described in the Materials and Methods section.
[d]Denotes values that are significantly different ($P \leq 0.05$) comparing butyrate treatment with controls based on nonpaired Student's *t*-test.

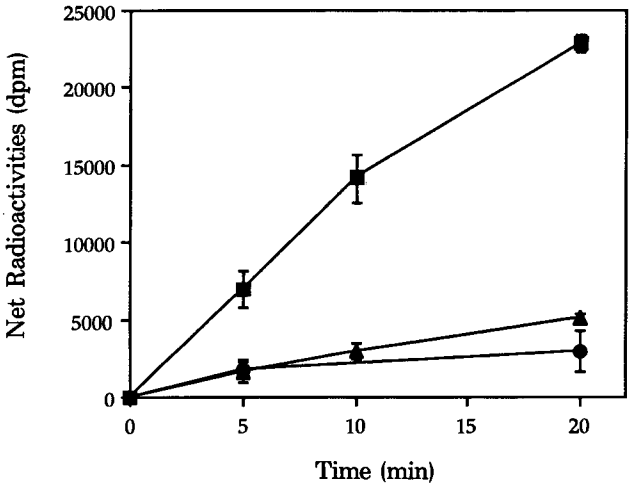

FIG. 1. Time-course of the net radioactivities of [^{3}H]arachidonic acid incorporated into phosphatidylcholine (■), phosphatidylinositol (▲), and phosphatidylethanolamine (●) of neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates using an *in vitro* assay as described in the Materials and Methods section. Homogenates from 1 × 10^6 cells, 1 μCi [^{3}H]arachidonic acid, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) were incubated at 37°C for the time periods indicated. Radioactivity of the lipids (dpm) is expressed as means ± SD from three determinations; dpm, disintegration per minute.

into TG, whereas LPC inhibited acyl incorporation into TG (Fig. 3). Prior to assay for arachidonoyl transfer activity *in vitro,* CB-II cells were exposed to butyrate for 35 h. Again, there was a significant increase in incorporation of labeled arachidonate into PC in butyrate pretreated cells (Table 4). Under this condition, the butyrate pretreated cells showed a decrease in labeling of TG instead.

Effect of sodium butyrate on lysophospholipid:acyl-CoA acyltransferase. The enzyme activity was assayed by incubating [^{14}C]arachidonoyl-CoA with cell homogenates, as well as in the presence of various classes of lysophospholipids. The results demonstrated that radioactivities were preferentially incorporated into PC (Fig. 4) indicating active LPC:arachidonoyl-CoA acyltransferase activity in CB-II. In the absence of LPC, sodium butyrate alone did not stimulate the incorporation into PC (data not shown). However when LPC (20 μM) was added to the incubation

TABLE 3

***In Vitro* Effect of Sodium Butyrate on [^{3}H]Arachidonic Acid Incorporation into Phospholipids of CB-II Homogenates[a,b,c]**

Phospholipid	Sodium butyrate (mM)			
	0	2.5	5.0	7.5
	(dpm)			
PI	12121 ± 1066	11018 ± 1503	10793 ± 2001	10587 ± 562
PC	17655 ± 500	21351 ± 909[d]	26427 ± 2369[d]	28811 ± 1584[d]
PE	8267 ± 311	7285 ± 856	9465 ± 1612	9553 ± 1887
Total	46875 ± 3575	50622 ± 6881[d]	63521 ± 2675[d]	69991 ± 7051[d]

[a]Radioactivity of the lipids (dpm) is expressed as means ± SD from three determinations. Abbreviations as in Table 1.
[b]Cells (1.2×10^7) were homogenized in Tris-HCl (pH 7.4). Homogenates from 1×10^6 cells (approximately 200 μg protein) were incubated with 1 μCi [^{3}H]arachidonic acid, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) at the indicated concentrations of sodium butyrate in a total of 1 mL at 37°C for 10 min.
[c]The methods for separation and measurement of the radioactivities of PI, PC and PE are described in the Materials and Methods section.
[d]Denotes values that are significantly different ($P \leq 0.05$) comparing butyrate treatment with controls based on nonpaired Student's *t*-test.

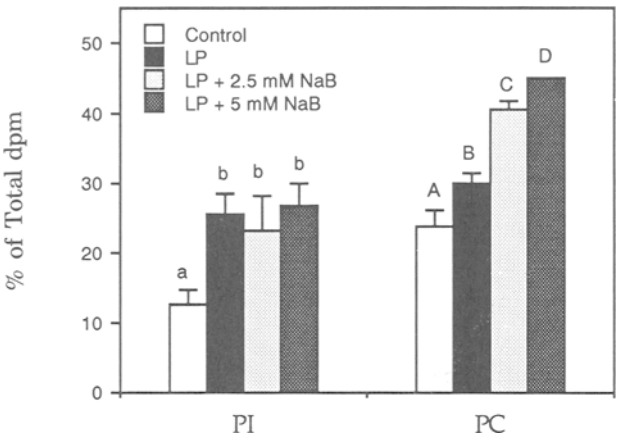

FIG. 2. Effect of sodium butyrate (NaB) and lysophospholipids (LP) on the incorporation of [^{3}H]arachidonic acid into phosphatidylinositol (PI) and phosphatidylcholine (PC) of neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates. Cell homogenates from 1×10^6 cell were incubated with 1 μCi [^{3}H]arachidonic acid, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4), and the indicated lysophospholipids (LP) at the indicated concentrations of NaB at 37°C for 10 min. The LP were 4 mM lysophosphatidylinositol for the synthesis of PI, and 20 mM lysophosphatidylcholine for the synthesis of PC. Radioactivity of the lipids (dpm) is expressed as % of total means ± SD from three determinations. Bars marked with different letters differ significantly ($P \leq 0.05$) when compared with controls (no NaB, no LP) based on nonpaired Student's *t*-test. Other abbreviations as in Figure 1.

TABLE 4

Incorporation of [^{3}H]Arachidonic Acid into Phospholipids and Triacylglycerols of Control and Sodium Butyrate Pretreated CB-II Cell Homogenates[a,b,c]

Phospholipid	Control	Pretreated
	(% of total)	
PI	23.6 ± 3.57	23.4 ± 1.34
PS	4.84 ± 1.35	4.73 ± 0.75
PC	33.8 ± 5.35	42.2 ± 1.60[d]
PEpl	10.4 ± 0.80	8.21 ± 2.12
PE	11.1 ± 1.14	11.3 ± 1.25
TG	16.2 ± 2.43	10.2 ± 0.15[d]
Total dpm	56418 ± 8278	74495 ± 9056

[a]Radioactivity of the lipids (dpm) is expressed as % of total dpm (mean ± SD) from three determinations. Abbreviations as in Table 1.

[b]Cells were cultured in DM 160 medium supplemented with 10% FBS in the absence or presence of 2.5 mM sodium butyrate for 35 h before obtaining homogenates for arachidonic acid incorporation experiments.

[c]Each incubation contained homogenates from 1×10^6 cells, 0.1 mM coenzyme A, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$, 0.3 mM dithiothreitol, 1 μCi [^{3}H]arachidonic acid, 50 mM Tris-HCl (pH 7.4) and was kept at 37°C for 10 min. The methods for separation and measurement of the radioactivities of PI, PS, PC, PEpl, PE and triacylglycerols (TG) are described in the Materials and Methods section.

[d]Denotes values that are significantly different ($P \leq 0.05$) comparing butyrate treatment with controls based on nonpaired Student's *t*-test.

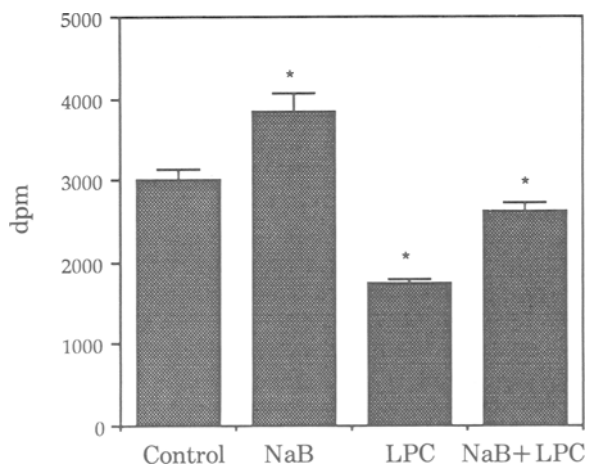

FIG. 3. Effect of NaB and lysophosphatidylcholine (LPC) on the incorporation of [^{3}H]arachidonic acid into triacylglycerols of neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates. Radioactivity of the lipids (dpm) is expressed as means ± SD from three determinations. An asterisk denotes values that are significantly different from controls (no NaB, no LPC) with $P \leq 0.05$ based on nonpaired Student's *t*-test. Homogenates from 1×10^6 cell were incubated with 1 μCi [^{3}H]arachidonic acid, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) in the presence or absence of 20 μM LPC and in the presence or absence of 2.5 mM NaB at 37°C for 10 min. Other abbreviations as in Figure 1.

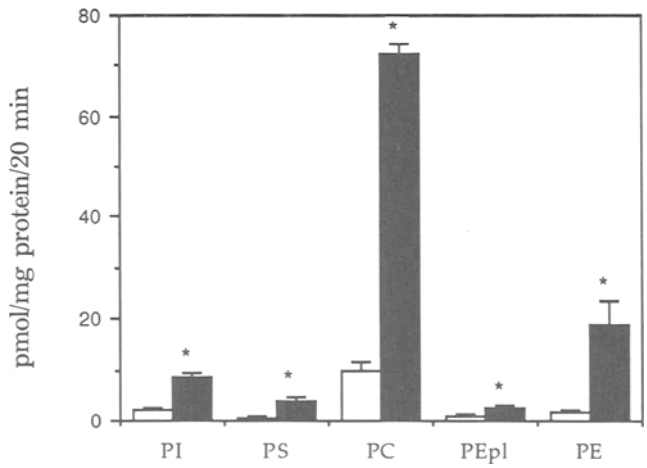

FIG. 4. Effect of lysophospholipids on the incorporation of [1-^{14}C]arachidonoyl-coenzyme A into PI, phosphatidylserine (PS), PC, ethanolamine plasmalogen (PEpl) and phosphatidyl-ethanolamine (PE) of neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates. Homogenates from 1×10^6 cells (approximately 220 μg of protein) were incubated with 50 nCi [1-^{14}C]arachidonoyl-coenzyme A and 50 mM Tris-HCl (pH 7.4) in the absence (□) or presence (■) of 4 μM lysophosphatidylinositol, or 4 μM lysophosphatidylserine or 20 μM lysophophatidylethanolamine, or 40 μM bovine brain lysophosphatidylserine, at 37°C for 20 min. An asterisk denotes values that are significantly different from controls (no LP) with $P \leq 0.05$ based on nonpaired Student's *t*-test. The methods for separation and measurement of the levels of PI, PS, PC, PEpl and PE are described in the Materials and Methods section. Other abbreviations as in Figure 2.

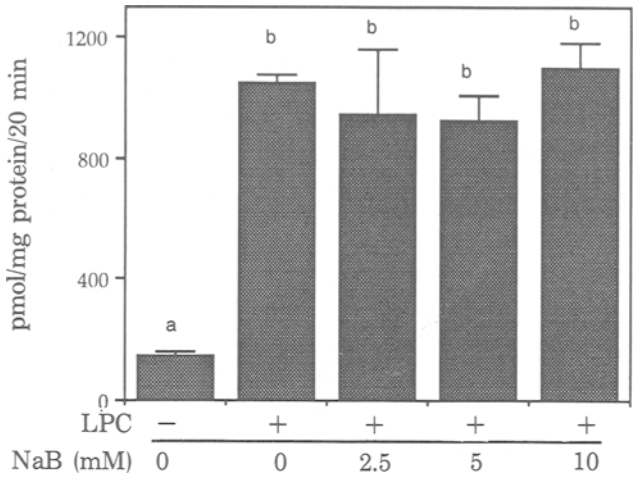

FIG. 5. Effect of NaB and LPC on incorporation of [1-^{14}C]-arachidonoyl-CoA into phosphatidylcholine of neonate rat cerebellum-derived clonal oligodendrocyte homogenates. Homogenates from 1×10^6 cells (approximately 220 μg of protein) were incubated with 50 nCi [1-^{14}C]arachidonoyl-coenzyme A in the absence (–) or presence (+) of 20 μM LPC at the indicated concentrations of NaB at 37°C for 20 min. Bars marked with letter b denote values that are significantly different from controls (bar marked with letter a) (no NaB, no LPC) with $P \leq 0.05$ based on nonpaired Student's *t*-tests. Abbreviations as in Figures 2 and 3.

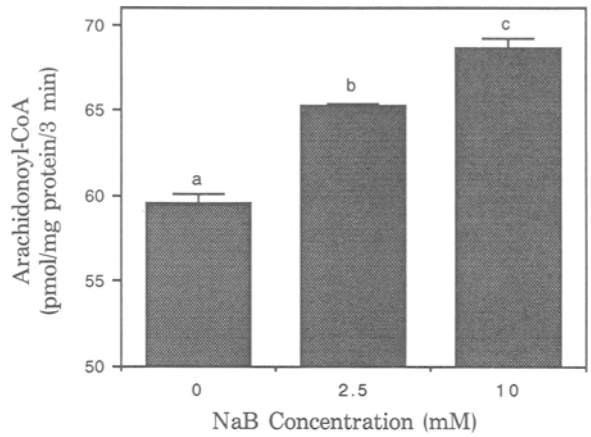

FIG. 7. Dose response relationship of the effect of sodium butyrate on the levels of arachidonoyl-CoA in neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates. Homogenates from 5×10^5 cells were incubated at 37°C with 50 nCi [^{14}C]arachidonic acid, 0.1 mM CoA, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) in the absence (0) or presence (2.5–10 mM) of sodium butyrate for three minutes. Arachidonoyl-CoA was extracted and measured as described in the Materials and Methods section. The values are means ± SD from three determinations. Bars marked with different letters differ significantly ($P \leq 0.05$) based on nonpaired Student's *t*-test. Abbreviations as in Figures 2 and 6.

system, there was a fivefold increase in labeling of PC. Interestingly, addition of sodium butyrate (2.5–7.5 mM) to the incubation system did not cause a further increase in labeling of PC (Fig. 5).

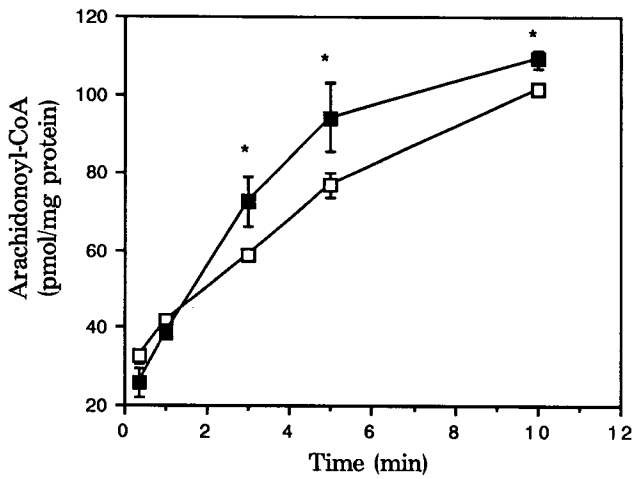

FIG. 6. Time course of synthesis of arachidonoyl-coenzyme A (CoA) in neonate rat cerebellum-derived clonal oligodendrocyte cell homogenates. Homogenates from 5×10^5 cells were incubated at 37°C with 50 nCi [^{14}C]arachidonic acid, 0.1 mM CoA, 0.3 mM dithiothreitol, 2.5 mM adenosine triphosphate, 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.4) in the absence (□) or presence (■) of 2.5 mM sodium butyrate for various time periods. The level of arachidonoyl-CoA at each time point was determined as described in the Materials and Methods section. The values are means ± SD from three determinations. An asterisk denotes values that are significantly different from controls (no butyrate) with $P \leq 0.05$ based on nonpaired Student's *t*-test.

Effect of sodium butyrate on acyl-CoA ligase. The effect of sodium butyrate (2.5–10 mM) on arachidonoyl-CoA synthesis was investigated. Initially, a time course study was carried out in which homogenates from 5×10^5 cells were incubated with [^{14}C]arachidonic acid in the absence or presence of 2.5 mM sodium butyrate at 37°C. As shown in Figure 6, the amount of arachidonoyl-CoA formed increased with time in the presence of sodium butyrate (2.5 mM). In a similar assay system, a dose dependent increase in the synthesis of arachidonoyl-CoA was observed in the incubation system containing 2.5 and 10 mM sodium butyrate (Fig. 7).

DISCUSSION

Although sodium butyrate is known to exert multiple effects on cells, the exact mechanism leading to morphological transformations and concomitant changes in membrane phospholipid profiles had not been elucidated previously. In our earlier study, the effect of butyrate on the morphology of CB-II cells, a clonal oligodendrocyte cell line, correlated with the ability of butyrate to enhance the incorporation of labeled arachidonic acid into PC and decrease its incorporation into PE (9). In the present study, the effect of butyrate on phospholipid metabolism was further studied by examining lysophospholipid acyltransferase and acyl-CoA ligase activities. These studies were prompted by the finding that sodium butyrate altered incorporation of labeled arachidonate. In general, polyunsaturated fatty acids are preferentially transferred into phospholipids by using the acylation–deacylation route, whereas saturated fatty acids are incorporated into phos-

pholipids *via* the *de novo* pathway (12,23). Thus, in agreement with earlier data (24,25), myristate was preferentially incorporated into PC of CB-II cells. Because butyrate did not alter the incorporation of labeled myristate into PC, it was reasonable to assume that butyrate does not affect *de novo* PC biosynthesis. Similarly, butyrate did not alter choline incorporation into PC. However, in experiments in which cells were incubated with labeled choline, butyrate caused a decrease in labeled LPC. As LPC is an intermediate in the acylation–deacylation process, a change in LPC further corroborates the action of butyrate on this cyclic process.

When cell homogenates were incubated with [^{3}H]arachidonate in the absence of exogenous lysophospholipids, there was a time-dependent increase in labeling of PC. This would imply that the membranes contained endogenous LPC as well as acyltransferase to incorporate [^{3}H]arachidonate into PC. The results in Figure 1 further indicate a preference for transfer of arachidonate to LPC, rather than LPI and LPE. This would be in agreement with the recent analyses of the phospholipids of ROC-1 oligodendroglia cells, which showed that more LPC (0.6 mol %) than LPI is present in these cells (26). Earlier studies had also indicated (27,28) that incubation of brain membranes with labeled arachidonic acid resulted in preferential transfer of arachidonic acid to LPC and LPI, rather than LPE and LPS. Although both LPC and LPI acyltransferases are present in CB-II cells, as indicated by the increase in transfer of labeled arachidonic acid into PC and PI in the presence of exogenous LPI and LPC, only the transfer to LPC was affected by butyrate. The reason for the selective effect of butyrate is not clear, but the data are consistent with the notion that different lysophospholipid acyltransferases are involved in the transfer of arachidonate to LPC and LPI (12) and that these acyltransferases respond differently to butyrate.

In the *in vitro* assay system, labeled arachidonic acid is first activated to its acyl-CoA derivative by acyl-CoA ligase prior to being transferred to lysophospholipids *via* lysophospholipid:acyl-CoA acyltransferase. There is evidence that these two enzymes operate in a cooperative manner (12,27). Consequently, it is possible that the effect of butyrate on the transfer of arachidonate to PC is related to a specific effect on one of the two enzymes or on a coupled mechanism between fatty acid activation and transfer of acyl-CoA to the lysophospholipid. By using [^{14}C]arachidonoyl-CoA as substrate, we demonstrated the lack of an effect of butyrate on the lysophospholipid:acyl-CoA acyltransferase.

In one experiment, butyrate also enhanced the incorporation of arachidonic acid into TG, and LPC inhibited the acyltransfer into TG. This agrees with the finding that there is a reciprocal regulatory effect of diacylglycerol and lysophospholipids on acyltransfer to TG and phospholipid in brain membranes (12). It is interesting to note that in homogenates from cells pretreated with sodium butyrate, the increase in labeling of PC was associated with a decrease in labeling of TG (Table 4). It remains to be seen whether the decrease in TG is associated with a decrease in the diacylglycerol pool. To examine whether butyrate-enhanced PC labeling was due to stimulation of CoA-ligase activity conversion of labeled arachidonate to its acyl-CoA derivative was measured in CB-II homogenates. As indicated in Figures 6 and 7, the results showed that the synthesis of arachidonoyl-CoA is stimulated by butyrate.

In conclusion, we have shown that sodium butyrate-induced CB-II cell differentiation and morphological transformations are accompanied by enhanced incorporation of arachidonic acid into PC. Furthermore, enhanced PC synthesis was shown to be due to stimulation of the acylation route. We also showed that butyrate specifically increased acyl-CoA ligase activity. These results provide some novel insights into the role of phospholipids in cell differentiation and growth.

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Stimulation of Phosphatidylglycerolphosphate Phosphatase Activity by Unsaturated Fatty Acids in Rat Heart

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Phosphatidylglycerolphosphate (PGP) synthase and PGP phosphatase catalyze the sequential synthesis of phosphatidylglycerol from cytidine-5′-diphosphate 1,2-diacyl-*sn*-glycerol (CDP-DG) and glycerol-3-phosphate. PGP synthase and PGP phosphatase activities were characterized in rat heart mitochondrial fractions, and the effect of fatty acids on the activity of these enzymes was determined. PGP synthase was observed to be a heat labile enzyme that exhibited apparent K_m values for CDP-PG and glycerol-3-phosphate of 46 and 20 μM, respectively. The addition of exogenous oleic acid to the assay mixture did not affect PGP synthase activity. PGP phosphatase was observed to be a heat labile enzyme, and addition of oleic acid to the assay mixture caused a concentration-dependent stimulation of PGP phosphatase activity. Maximum stimulation (1.9-fold) of enzyme activity was observed in the presence of 0.5 mM oleic acid, but the stimulation was slightly attenuated by the presence of albumin in the assay. The presence of oleic acid in the assay mixture caused the inactivation of PGP phosphatase activity to be retarded at 55°C. Stimulation of PGP phosphatase activity was also observed with arachidonic acid, whereas taurocholic, stearic and palmitic acids did not significantly affect PGP phosphatase activity. The activity of mitochondrial phosphatidic acid phosphohydrolase was not affected by inclusion of oleic acid in the incubation mixture. We postulate that unsaturated fatty acids stimulate PGP phosphatase activity in rat heart.

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Phosphatidylglycerol (PG) is an important intermediate in the biosynthesis of cardiolipin (CL); the biosynthetic pathway of PG in mammalian tissues was first elucidated by Kiyasu *et al.* (1). In the isolated rat heart, the biosynthesis of new PG proceeds *via* the cytidine-5′-diphosphate (CDP)-diglyceride pathway (2). Glycerol is taken up by the heart and is phosphorylated to glycerol-3-phosphate (3), which is rapidly acylated to form phosphatidic acid (PA) (2). PA is converted to CDP 1,2-diacyl-*sn*-glycerol (CDP-DG) by PA:CTP cytidylyltransferase (4), the rate-limiting step of PG and CL biosynthesis in isolated rat heart (2). CDP-DG is condensed with glycerol-3-phosphate to form phosphatidylglycerolphosphate (PGP) catalyzed by PGP synthase (1,2). PGP is then dephosphorylated by PGP phosphatase to form PG (1,2). The activities of the latter two enzymes are confined to the mitochondrial fraction in mammalian tissues (1,2,5).

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Abbreviations: CDP-DG, cytidine-5′-diphosphate 1,2-diacyl-*sn*-glycerol; CL, cardiolipin; CTP, cytidine-5′-triphosphate; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate.

There are few studies on the regulation of PGP synthase and PGP phosphatase activities in mammalian tissues. PGP synthase was partially purified and characterized from pig liver by McMurray and Jarvis (6). Phosphatidylethanolamine was shown to stimulate the activity of the partially purified enzyme. MacDonald and McMurray (7) partially purified and characterized PGP phosphatase from rat liver. PA (NH_4^+-salt) was shown to inhibit the phosphatase activity whereas PA (Na^+-salt) stimulated the activity. Thus, there is a precedent for lipid modulation of the activity of PGP synthase and PGP phosphatase.

Fatty acids are potent stimulators of several enzymes involved in phospholipid biosynthesis. For example, PA phosphohydrolase, the committed step of DG production, is stimulated by oleic acid (8). In addition, oleic acid stimulates the activity of CTP:phosphocholine cytidylyltransferase, the rate-limiting step of phosphatidylcholine biosynthesis (9,10). This stimulation of enzyme activity was associated with increases in phosphatidylcholine biosynthesis. The heart has a tremendous capacity to take up and utilize fatty acids (11). It is not known if fatty acids modulate PGP synthase and PGP phosphatase activities in the heart. In this paper we provide evidence that rat heart mitochondrial PGP phosphatase activity is stimulated by unsaturated fatty acids.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (200–250 g) were obtained from Charles River Canada Inc. (St. Constante, Quebec, Canada). [^{14}C(*U*)]Glycerol-3-phosphate was purchased from DuPont Canada, Inc. (Mississauga, Ontario, Canada). Phosphatidyl[^{14}C(*U*)]glycerol-3-phosphate was synthesized as described previously (2). Thin-layer plates (silica gel 60, 0.25 mm thick) were obtained from Canlab Division of Baxter Co. (Winnipeg, Manitoba, Canada). Biochemicals used in this study were of analytical grade and purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Edmonton, Alberta, Canada).

Preparation of rat heart mitochondrial fractions. Animals were sacrificed by decapitation, and the heart was rapidly removed and placed in ice-cold saline. Exogenous blood was removed by perfusion of the heart in the Langendorff mode (12) for 2 min with ice-cold saline. The heart was then cut into small pieces, and a 10% homogenate was prepared in 0.25 M sucrose, 0.145 M NaCl, 5 mM Tris-HCl, pH 7.4, using a Polytron (Model PT10-35; Kinematika, Lucerne, Switzerland) homogenizer. The homogenate was centrifuged at 1,000 × *g* for 5 min (Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS-34 rotor; DuPont Canada Inc.). The pellet was

discarded, and the resulting supernatant was centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended in 1 mL of homogenizing buffer with a tight-fitting Dounce tissue grinder and used as the source of mitochondrial fraction for assay of PGP synthase, PGP phosphatase and PA phosphohydrolase. The supernatant from the 10,000 × *g* separation was centrifuged at 105,000 × *g* for 60 min (Beckman Model L8-70 Ultracentrifuge with Ti 70.1 rotor; Beckman Instruments, Inc., Mississauga, Ontario, Canada). The pellet was resuspended in 1 mL of homogenizing buffer with a tight-fitting Dounce tissue grinder and used as the source of the microsomal fraction. The mitochondrial fraction was contaminated with 10% of microsomal material, and the microsomal fraction contained 5% mitochondrial material (13).

Preparation of fatty acids. A stock 80 mM oleic acid solution was prepared by adding 1.13 g oleic acid to 4.8 mL of 1 M KOH. The mixture was heated to 60°C, and the volume was made up to 50 mL with distilled water. The oleic acid stock solution may be repeatedly frozen for storage at –20°C and upon thawing is a clear liquid. Oleic acid prepared in this fashion was shown to stimulate cytidine-5′-triphosphate (CTP):phosphocholine citidylyltransferase activity *in vitro* (9,10). Sodium taurocholate was dissolved in water, and other fatty acids were dissolved in water with KOH, vortexed and sonicated until a clear suspension was obtained prior to addition to the PGP phosphatase assay mixture.

Assay of PGP synthase, PGP phosphatase and PA phosphohydrolase. PGP synthase was assayed by determining the conversion of [^{14}C(*U*)]glycerol-3-phosphate to phosphatidyl[^{14}C(*U*)]glycerol-3-phosphate (14). The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 10 mM freshly prepared ß-mercaptoethanol, 0.5 mM [^{14}C(*U*)]glycerol-3-phosphate (specific activity, 12,500 dpm/nmol), 0.2 mM CDP-DG dissolved in 10 mM Triton X-100 and 50–100 µg of mitochondrial protein to a final volume of 90 µL. The reaction was initiated by the addition of 10 µL of 0.1 M $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30°C for 10 min and terminated by the addition of 0.5 mL of 0.1 M HCl in methanol. One mL of chloroform and 1.5 mL of 1 M $MgCl_2 \cdot 6H_2O$ was added to the mixture. The suspension was vortexed and centrifuged for 5 min at full speed in a clinical centrifuge (Model TJ-6; Beckman Instruments Inc.). The upper phase and interphase were removed by suction, and the organic phase was dried under nitrogen gas and resuspended in 50 µL of chloroform/methanol (2:1, vol/vol). A 25-µL aliquot of the resuspended organic phase was placed on an oxalate-treated thin-layer plate with PGP standard and developed in a solvent system containing chloroform/methanol/HCl (87:13:0.2, by vol). The thin-layer plates were prepared by soaking the plates in 0.5 M oxalic acid and air drying overnight prior to use. The iodine stained fractions corresponding to PGP were removed, and radioactivity in these fractions was determined. In some experiments 0.5–2.0 mM oleic acid was included in the incubation mixture.

PGP phosphatase was assayed by determining the conversion of phosphatidyl[^{14}C(*U*)]glycerol-3-phosphate to phosphatidyl[^{14}C(*U*)]glycerol (7). The reaction mixture contained 50 mM Tris-maleate (pH 6.5), 10 mM freshly prepared ß-mercaptoethanol, 0.5–1.5 µM phosphatidyl[^{14}C(*U*)]glycerol-3-phosphate (specific activity, 10,000 dpm/nmol) and 0.1 mg of mitochondrial protein to a final volume of 100 µL. The reaction mixture was incubated at 37°C for 5 min and terminated by the addition of 2 mL of chloroform/methanol/HCl (100:100:0.6, by vol). One mL of chloroform and 1.5 mL of 0.73% NaCl were added to facilitate phase separation. The tubes were centrifuged in a clinical centrifuge as described above, and the organic phase was evaporated under nitrogen gas. The organic phase was resuspended in 25 µL of chloroform/methanol (2:1, vol/vol) and 20 µL placed on a oxalate-treated plate with PG standard and was developed in the solvent system described above for separation of PGP. The iodine stained fractions corresponding to PG were removed and radioactivity in these fractions determined. In some experiments, 0.125 mM albumin (Sigma A-2153, Sigma Chemical Co.) and 0.5 mM fatty acid were added to the incubation mixture.

PA phosphohydrolase was assayed by determining the conversion of 1,2-diacyl-*sn*-[1,3-^{3}H]glycerophosphoric acid to 1,2-diacyl-*sn*-[1,3-^{3}H]glycerol (15). The incubation was performed in tubes which had previously been treated with dimethyldichlorosilane, 2% in 1,1,1-trichloroethane, to prevent the PA substrate from adhering to the glass tubes. The reaction mixture contained 100 mM Tris-HCl, pH 7.4, 1 mM dithioerythritol, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 0.4 mM phosphatidylcholine and 0.6 mM 1,2-diacyl-*sn*-[1,3-^{3}H]glycerophosphoric acid (specific activity, 0.25 µCi/pmol) in a total volume of 90 µL. The reaction mixture was pre-incubated for 5 min at 37°C, then 10 µL of 0.1 M $MgCl_2 \cdot 6H_2O$ was added to start the reaction. The mixture was incubated at 37°C for 60 min and terminated by the addition of 2 mL of chloroform/methanol (2:1, vol/vol). One mL of water was added to facilitate phase separation. The tubes were centrifuged in a clinical centrifuge as described earlier, and the organic phase was evaporated under nitrogen gas. The organic phase was resuspended in 25 µL of chloroform/methanol (2:1, vol/vol) and 20 µL placed on a thin-layer plate with standard 1,2-diacyl-*sn*-glycerol and developed in a solvent system containing petroleum ether/diethyl ether/acetic acid (60:40:1, by vol). The iodine stained fractions corresponding to 1,2-diacyl-*sn*-glycerol were removed and the radioactivity in these fractions determined. In some experiments, 0.2 mg albumin and 0.5 mM oleic acid were added to the incubation mixture.

*Synthesis of 1,2-diacyl-*sn*-[1,3-^{3}H]glycerophosphoric acid.* The 1,2-diacyl-*sn*-[1,3-^{3}H]glycerophosphoric acid was synthesized by perfusing two rat hearts with 100 µCi of 0.1 µM [1,3-^{3}H]glycerol for 15 min and extracting the lipids as described previously (2). PA was separated from other lipids on oxalate-treated plates using the solvent system described for the separation of PGP and PG. The plate was covered with cellophane, except for a standard lane containing PA, and stained with iodine. Silica gel corresponding to the location of the PA

standard was removed, and PA was extracted from the silica gel by the method of Arvidson (16).

Other analytical procedures. Protein was determined by the method of Bradford (17). Radioactivity was determined by liquid scintillation counting (Beckman Model LS 3801 Scintillation Counter; Beckman Instruments Inc.). Student's *t*-test was used for the statistical analysis of data. The level of significance was defined as $P < 0.05$.

RESULTS

Characterization of PGP synthase and PGP phosphatase activities. PGP synthase activity was assayed in mitochondrial fractions isolated from rat heart. PGP synthase activity was linear with time and protein concentration when 10 mM Mg^{+2} was included in the incubation mixture. Product identification revealed approximately 70–75% of radioactivity associated with PG and 20–25% with PGP (data not shown). The conversion of PGP to PG was due to the presence of PGP phosphatase activity in rat heart mitochondria (2). Less than 1% of radioactivity was associated with CL and PA. Mitochondrial fractions were incubated at 55°C for various periods of time prior to assay of PGP synthase activity. As seen in Figure 1A, the PGP synthase lost 26% of its activity by 1 min and 70% of its activity by 5 min of preincubation at 55°C. Thus, cardiac PGP synthase is a heat labile enzyme. When the concentration of CDP-DG was held constant at 0.2 mM and the concentration of glycerol-3-phosphate was varied, PGP synthase activity increased with increasing concentration of glycerol-3-phosphate. A double reciprocal plot of activity *vs.* glycerol-3-phosphate concentration revealed an apparent K_m for glycerol-3-phosphate of 20 μM (Fig. 1B). When the concentration of glycerol-3-phosphate was held constant at 0.5 mM and the concentration of CDP-PG was varied, PGP synthase activity increased with increasing concentration of CDP-DG. The increase was maximum at 0.2 mM CDP-PG. A double reciprocal plot of activity *vs.* CDP-DG concentration revealed an apparent K_m for CDP-DG of 46 μM (Fig. 1C). Less than 10% of the activity of the mitochondrial PGP synthase was observed in the 105,000 × *g* fraction of rat heart homogenates.

PGP phosphatase was assayed in mitochondrial fractions isolated from rat heart. The incubation mixture contained low substrate concentrations because high PGP concentrations (100 μM) are inhibitory to the enzyme (7). With 0.5 μM PGP in the assay, substrate conversion to PG was typically 20–25%. When mitochondrial fractions were incubated in the presence of various concentrations of PGP, the PGP phosphatase activity was increased with increasing concentrations of PGP in the assay mixture (Fig. 2A). Mitochondrial fractions were incubated at 55°C for various periods of time prior to assay of PGP phosphatase activity in the presence of 0.5 μM PGP. By 1, 2.5 and 5 min of preincubation at 55°C, 59, 88 and 95%, respectively, of the enzyme activity was lost (Fig. 2B). Thus, cardiac PGP phosphatase is a heat labile enzyme. Less than 10% of the activity was observed in the 105,000 × *g* fraction of rat heart homogenates.

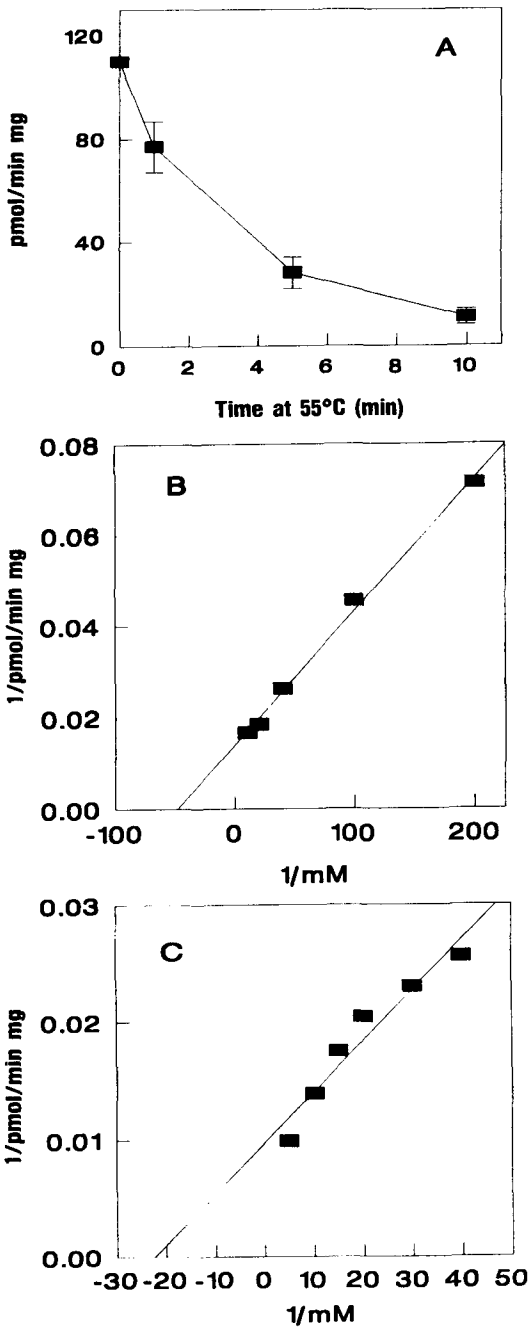

FIG. 1. Heat inactivation profile and kinetics of phosphatidylglycerolphosphate (PGP) synthase. A, A 0.1-mg aliquot of mitochondrial fraction was preincubated for up to 10 min at 55°C and PGP synthase activity subsequently determined. Each value represents the mean ± SD of three separate experiments, each of which was assayed in duplicate. B, PGP synthase activity was determined at various concentrations of glycerol-3-phosphate in the presence of 0.2 mM cytidine-5′-diphosphate 1,2-diacyl-*sn*-glycerol (CDP-DG). A double reciprocal plot of glycerol-3-phosphate concentration *vs.* activity is shown. Each value represents the mean of two separate experiments, each of which was assayed in duplicate. C, PGP synthase was assayed in the presence of various concentrations of CDP-DG in the presence of 0.5 mM glycerol-3-phosphate. A double reciprocal plot of CDP-DG concentration *vs.* activity is shown. Each value represents the mean of two separate experiments, each of which was assayed in duplicate.

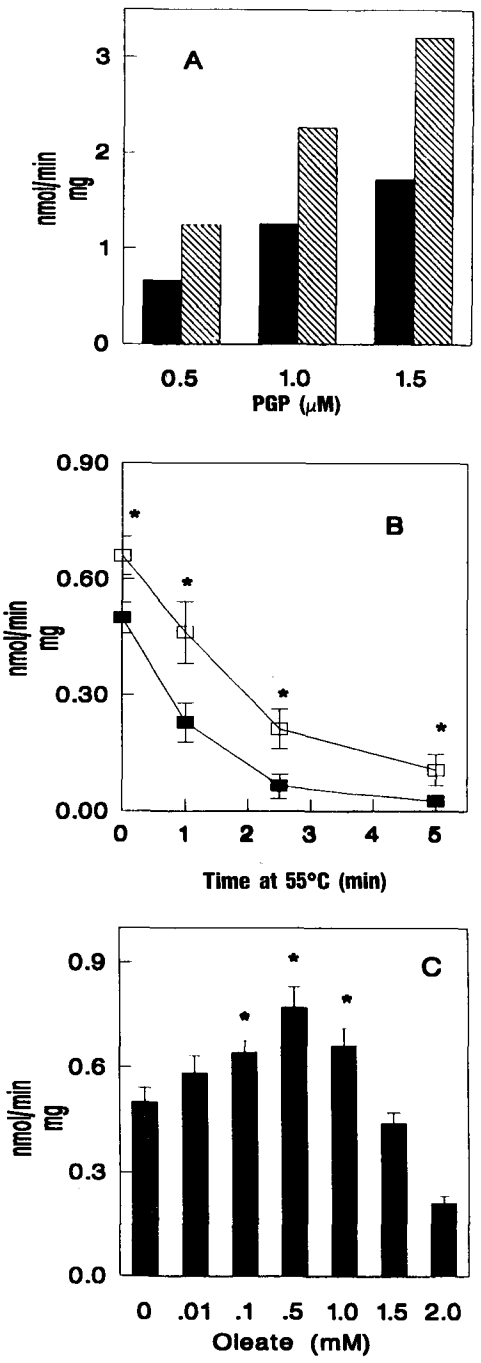

FIG. 2. Heat inactivation profile and effect of oleic acid on phosphatidylglycerolphosphate (PGP) activity. A, PGP phosphatase activity was determined in the absence (solid columns) or presence (hatched columns) of 0.5 mM oleic acid in the incubation mixture at various concentrations of PGP. Each value represents the mean of two separate experiments, each of which was assayed in duplicate. B, A 0.1-mg aliquot of mitochondrial fraction was preincubated for up to 5 min at 55°C, then PGP phosphatase activity was determined in the absence (closed squares) or presence (open squares) of 1.0 mM oleic acid. Each value represents the mean ± SD of three experiments, each of which was assayed in duplicate. C, PGP phosphatase activity was determined in the absence or presence of various concentrations of oleic acid. Each value represents the mean ± SD of three experiments, each of which was assayed in duplicate. *, $P < 0.05$.

Effect of fatty acids on PGP synthase, PGP phosphatase and PA phosphohydrolase activities. To determine if fatty acids affected the activity of PGP synthase, the enzyme activity was determined in isolated rat heart mitochondrial fractions in the absence or presence of 0.5–2.0 mM oleic acid in the assay mixture. PGP synthase activity was unaffected at 0.5–2.0 mM oleic acid concentrations. In addition, oleic acid did not affect the activity of PGP synthase in the presence of low or high glycerol-3-phosphate or CDP-DG concentrations (data not shown).

To determine the effect of fatty acids on PGP phosphatase activity, isolated mitochondrial fractions were incubated in the absence or presence of 0.1–2.0 mM oleic acid in the incubation mixture. The presence of 0.01–1.0 mM oleic acid in the incubation mixture caused a stimulation of PGP phosphatase activity (Fig. 2C). The stimulation was most pronounced at 0.5 mM. Higher concentrations of oleic acid (1.5–2.0 mM) inhibited PGP phosphatase activity. To determine the kinetics of stimulation of PGP phosphatase activity by oleic acid, PGP phosphatase activity was determined in the presence of increasing PGP concentrations in the absence or presence of 0.5 mM oleic acid in the incubation mixture. The presence of oleic acid caused a 1.9-fold stimulation of PGP phosphatase activity at all PGP concentrations (Fig. 2A). To determine if oleic acid altered the heat inactivation of PGP phosphatase, PGP phosphatase activity was determined in the absence or presence of 1.0 mM oleic acid in mitochondrial fractions that were preincubated for various times at 55°C. When 1.0 mM oleic acid was present in the assay mixture, PGP phosphatase activity was decreased only 29, 68 and 83% in mitochondrial fractions that had been preincubated for 1, 2.5 and 5 min at 55°C, respectively (Fig. 2B). Thus, in the presence of oleic acid, the inactivation of PGP phosphatase by heat denaturation was retarded.

Because fatty acids are associated with fatty acid binding proteins *in vivo* (18), we determined the effect of fatty acid bound to albumin on PGP phosphatase activity. A fatty acid/albumin ratio of 4:1 was used as albumin contains six to eight fatty acid binding sites per molecule (19). PGP phosphatase activity was determined in isolated rat heart mitochondrial fractions in the absence or presence of 0.125 mM albumin in the incubation mixture or in the presence of 0.125 mM albumin and 0.5 mM oleic acid. The presence of albumin in the incubation mixture resulted in a 20% decrease in PGP phosphatase activity (Fig. 3A). Thus, the albumin probably removes stimulatory lipids from the membrane. In the presence of 0.5 mM oleic acid and 0.125 mM albumin, PGP phosphatase activity was stimulated 36% compared to incubations that contained albumin alone (Fig. 3A). Thus, oleic acid stimulated PGP phosphatase activity in the presence of a fatty acid binding protein.

The effect of other fatty acids on PGP phosphatase activity was determined. PGP phosphatase activity was assayed in the absence or presence of both saturated and unsaturated fatty acids with differing chainlength and in the presence of the anionic bile salt taurocholic

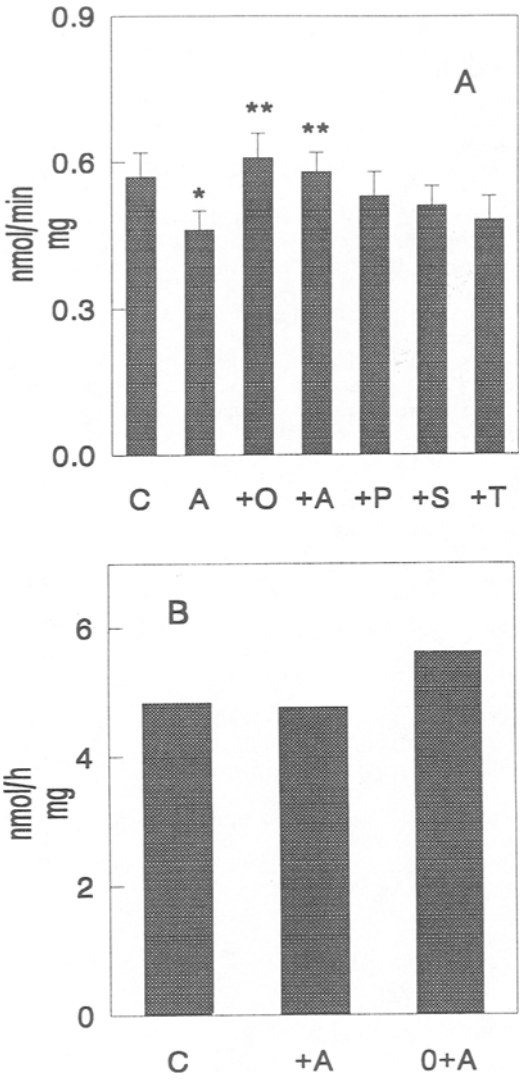

FIG. 3. Effect of albumin and fatty acids on phosphatidylglycerolphosphate (PGP) phosphatase and phosphatidic acid (PA) phosphohydrolase activities. A, PGP phosphatase activity was determined in the absence (C) or presence (A) of albumin or in the presence of albumin plus 0.5 mM oleate (+O), arachidonate (+A), palmitate (+P), stearate (+S) or taurocholate (+T). Each point represents the mean ± SD of three separate experiments, each of which was assayed in duplicate. *, $P < 0.05$, C *vs.* A; **, $P < 0.05$, A *vs.* +O, A *vs.* +A. B, PA phosphohydrolase activity was assayed in the absence (C) or presence (+A) of albumin or 0.5 mM oleic acid bound to albumin (O + A). Each value represents the mean of two separate experiments, each of which was assayed in duplicate. An 11–14% variation was observed between duplicate samples.

acid. Arachidonic acid stimulated PGP phosphatase activity, whereas the presence of taurocholic, stearic and palmitic acids in the assay mixture did not significantly affect PGP phosphatase activity (Fig. 3A).

Fatty acids are known to stimulate PA phosphohydrolase activity by promoting the translocation of the enzyme from the cytosol to the endoplasmic reticulum (8). As a control, we determined the effect of oleic acid on PA phosphohydrolase activity in isolated rat heart mitochondrial fractions. PA phosphohydrolase activity was determined in the absence or presence of 0.2 mg albumin or 0.2 mg albumin and 0.5 mM oleic acid in the incubation mixture. In the presence of albumin or albumin and oleic acid, PA phosphohydrolase activity was not affected (Fig. 3B).

DISCUSSION

The objective of this study was to investigate if the heart PGP synthase and PGP phosphatase activities were modulated by fatty acid. It is clear from this study that unsaturated fatty acids stimulate PGP phosphatase activity in rat heart mitochondria. The stimulation of PGP phosphatase by oleic acid in the absence or presence of albumin coupled with the retarded heat denaturation of the enzyme activity in the presence of oleic acid support this conclusion.

Lipid modulation of enzyme activity has been regarded as a plausible mechanism for the control of phospholipid and triacylglycerol biosynthesis. Oleic acid has been shown to stimulate the activities of PA phosphohydrolase and CTP:phosphocholine cytidylyltransferase, enzymes involved in triacylglycerol and phosphatidylcholine biosynthesis (8–10). In our study, free oleic acid and oleic acid bound to albumin stimulated the activity of heart mitochondrial PGP phosphatase, an enzyme involved in PG and CL biosynthesis. Remarkably, oleic acid appeared to retard the heat inactivation of the PGP phosphatase. However, because the rate of inactivation of PGP phosphatase in control and oleic acid containing incubations was identical, the presence of oleic acid likely promotes enzyme activation. In contrast, high concentrations of free oleic acid inhibited PGP phosphatase activity. This was probably due to an alteration in the enzyme lipid associations required for enzyme catalysis.

The heart PGP phosphatase activity was stimulated by long-chain fatty acids consistent with fatty acid stimulation of other lipogenic enzymes (9,20,21). However, stimulation was significant and most pronounced with unsaturated fatty acids similar to that observed for phospholipase D activation by fatty acids (21). In a previous study, ionic and nonionic detergents were shown to inhibit the partially purified PGP phosphatase from rat liver in the absence of albumin in the incubation mixture (7). In our study, the anionic bile detergent taurocholic acid did not affect the heart PGP phosphatase activity at a concentration in which unsaturated fatty acids were stimulatory when albumin was present in the incubation mixture. This observation suggests that the effect of unsaturated fatty acids on the stimulation of the heart PGP phosphatase was not due to their detergent-like properties. In addition, it was unlikely that low concentrations of oleic acid markedly altered the physical state of the PGP phosphatase and the surrounding membrane structure since the kinetic properties of PGP synthase were not affected by oleic acid.

It was previously postulated that PGP phosphatase and PA phosphohydrolase were the same enzyme in rat lung lamellar fractions (22). However, it was suggested

by Chang and Kennedy (23) that *Escherichia coli* membrane PGP phosphatase activity toward PA may be due to contamination by some other enzyme. In fact, PA phosphohydrolase purified from yeast cell membranes was shown not to dephosphorylate PGP, nor did PA competitively inhibit PGP hydrolysis (24). Rat liver plasma membrane PA phosphohydrolase activity was shown to be stimulated by oleic acid (25). In contrast, oleic acid did not affect the activity of the heart mitochondrial PA phosphohydrolase. Furthermore, the addition of albumin to the incubation mixture did not affect PA phosphohydrolase activity. Thus, the activities of PA phosphohydrolase and PGP phosphatase in rat heart mitochondria appear to be distinct.

Severe diabetes (26) and hypoxia (27) are metabolic conditions that predispose tissues to high concentrations of fatty acids and may affect the regulation of metabolic enzymes. Indeed, rat liver PA phosphohydrolase activity is increased in diabetes, and this may account for the increases in triacylglycerols accumulated in the liver and in the systemic circulation in this disease (8,26). The heart has a large capacity to take up and utilize fatty acids (11). Thus, under altered metabolic conditions, such as severe diabetes or hypoxia, it is possible that the heart PGP phosphatase activity may thus be regulated *in vivo*. Significant levels of PGP have been detected in brain (28,29). However, PGP is found in only limited amounts in other tissues *in vivo* (1). Thus, an important question is whether the heart PGP phosphatase is, indeed, stimulated by unsaturated fatty acids *in vivo*. As low concentrations of free oleic acid and unsaturated fatty acid bound to albumin stimulated the heart PGP phosphatase activity at low PGP concentration, it is possible that modulation of the activity of this enzyme and, thus, heart polyglycerophospholipid biosynthesis at the level of the PGP phosphatase may occur. We are currently investigating this possibility in isolated perfused rat hearts.

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Effect of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on Lipid-Metabolizing Enzymes in Obese Rat Liver

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This study was designed to examine whether n-3 and n-6 polyunsaturated fatty acids at a very low dietary level (about 0.2%) would alter liver activities in respect to fatty acid oxidation. Obese Zucker rats were used because of their low level of fatty acid oxidation, which would make increases easier to detect. Zucker rats were fed diets containing different oil mixtures (5%, w/w) with the same ratio of n-6/n-3 fatty acids supplied either as fish oil or arachidonic acid concentrate. Decreased hepatic triacylglycerol levels were observed only with the diet containing fish oil. In mitochondrial outer membranes, which support carnitine palmitoyltransferase I activity, cholesterol content was similar for all diets, while the percentage of 22:6n-3 and 20:4n-6 in phospholipids was enhanced about by 6 and 3% with the diets containing fish oil and arachidonic acid, respectively. With the fish oil diet, the only difference found in activities related to fatty acid oxidation was the lower sensitivity of carnitine palmitoyltransferase I to malonyl-CoA inhibition. With the diet containing arachidonic acid, peroxisomal fatty acid oxidation and carnitine palmitoyltransferase I activity were markedly depressed. Compared with the control diet, the diets enriched in fish oil and in arachidonic acid gave rise to a higher specific activity of aryl-ester hydrolase in microsomal fractions. We suggest that slight changes in composition of n-3 or n-6 polyunsaturated fatty acids in mitochondrial outer membranes may alter carnitine palmitoyltransferase I activity.

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Fish oils (FO) are known to lower the risk of cardiovascular disease partly be reducing plasma triacylglycerols (TAG) (1–3). The effect can be mainly attributed to the amounts of long-chain polyunsaturated fatty acids (PUFA) of the n-3 series, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in marine oils. It was recently demonstrated that rats fed dietary FO rather than olive oil have a lower rate of very low density lipoprotein (VLDL) TAG production in liver and a lower liver concentration of total lipids and TAG (4) suggesting that FO stimulates fatty acid (FA) oxidation and/or impairs TAG synthesis. Indeed, previous studies had shown that n-3 FA are preferentially directed toward β-oxidation pathways in liver cells (5) and mitochondria (6). Moreover, dietary FO has been found to reduce the activity of enzymes involved in FA and TAG synthesis (5,7–10) and to stimulate the FA oxidative pathway in the liver (11).

In rats fed high FO diets, lowered circulating TAG levels have been associated with peroxisome proliferation and a concomitant increase in peroxisomal oxidation (12). However, lower levels of FO consumption (below 15%, w/w of the diet) do not result in significant peroxisomal proliferation (13,14). In Syrian hamsters consuming marine oils at levels below the threshold for hepatic peroxisomal proliferation, the enhanced activity of mitochondrial carnitine palmitoyltransferase (CPT I) has suggested that the decreased hepatic output of TAG may be related to increased mitochondrial FA oxidation (15). In addition, dietary FO have been shown to enhance CPT I activity in rats (14) and mice (16).

The characteristics of FA oxidation are often assessed by measuring series of parameters, including ketone body production and the activity of total CPT (both forms) or CPT I (overt form alone) with either palmitoyl-CoA or palmitate as substrate in animals fed diets containing high amounts of oils (9–11). Since different levels of the same oil in the diet may influence lipogenesis differentially (17,18) and thus have varying effects on mitochondrial FA oxidation (19), the present study was designed to test the validity of previous findings by using diets containing only a moderate percentage of oil mixture (5%, w/w) with the same ratio of n-6/n-3 FA but differing in their contents of PUFA, which were supplied either from FO (n-3 PUFA) or arachidonic acid (AA) concentrate (n-6 PUFA). Obese Zucker rats were chosen because they are known to have a lower rate of hepatic FA oxidation compared to their lean littermates (20,21) and thus were expected to display a relatively greater enhancement of oxidative pathways after feeding the diets enriched in PUFA. It had also been shown that the proportion of 20:4n-6 [which is lower in liver phospholipids (PL) of obese rats] was enhanced after hypolipidemic treatment (22) concomitantly with enhanced mitochondrial FA oxidation (23). These previous observations prompted us to study the effects of direct supplementation of the diet with dietary n-6 and n-3 PUFA on selected mitochondrial activities and on the lipid composition of mitochondrial outer membranes (OM) in which CPT I (which catalyzes a controlling step in the pathway of β-oxidation) resides. In addition, the activity of microsomal aryl-ester hydrolase, which is assumed to control the intracellular pool of acyl-CoA esters (13,14), was monitored, as its induction could reflect increased acyl-CoA levels.

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Abbreviations: AA, arachidonic acid, 20:4n-6; CGLC, capillary gas–liquid chromatography; CPT I, carnitine palmitoyltransferase I; DHA, docosahexaenoic acid, 22:6n-3; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N'*-tetraacetic acid; EPA, eicosapentaenoic acid, 20:5n-3; FA, fatty acids; FO, fish oil(s); OM, outer membrane(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFAOS, peroxisomal fatty acid oxidizing system; PL, phospholipid(s); PPMF, percoll-purified mitochondrial fraction; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol(s); Tris, *tris*(hydroxymethyl)aminomethane; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. L-[*Methyl*-^{3}H]carnitine was obtained from the Amersham Radiochemical Centre (Amersham, Bucks., United Kingdom). Unlabeled L-carnitine was from Dr. C. Cavazza of Sigma-Tau (Pomezia, Italy). Fatty acid-free albumin (Fraction V) used in the homogenization mixture was from Paesel-Lorei (Frankfurt, Germany). Percoll was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Coenzymes, CoA derivatives and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Standard lipids were obtained from Nu-Chek-Prep (Elysian, MN). The chemicals from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade.

Animals. Obese (fa/fa) male Zucker rats were bred in the Centre de Sélection et d'Elevage d'Animaux de Laboratoire, CNRS (Orléans-la-Source, France). They were five-weeks-old on arrival at the Station de Recherches de Nutrition (INRA-CRJ, Jouy-en-Josas, France) and were housed in stainless-steel cages in a well-ventilated room maintained at 22 ± 2°C on a 12 h light/dark cycle. All animals received a control diet containing (g/kg): casein, 220; DL-methionine, 1.6; sucrose, 218; corn starch, 440; cellulose powder, 20; vitamin mixture, 10 (choline chloride, 0.75 g; thiamine, 10 mg; riboflavin, 10 mg, pyridoxin · HCl, 10 mg; nicotinic acid, 45 mg; Ca pantothenate, 30 mg; biotin, 0.2 mg; folic acid, 0.9 mg; cyanocobalamin, 13.5 µg; inositol, 50 mg; retinoic acid, 45 mg; cholecalciferol, 2.5 mg; ascorbic acid, 450 mg; α-tocopherol, 50 mg; menadione, 22.5 mg; *p*-aminobenzoic acid, 50 mg; and sucrose to 10 g); mineral mixture, 40 [$CaHPO_4 \cdot 2\ H_2O$, 15.2 g; K_2HPO_4, 9.6 g; $CaCO_3$, 7.24 g; NaCl, 2.8 g; MgO, 0.8 g; $MgSO_4 \cdot 7\ H_2O$, 3.6 g; $FeSO_4 \cdot 7\ H_2O$, 0.28 g; $ZnSO_4 \cdot H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.2 g; $CuSO_4 \cdot 5\ H_2O$, 40 mg; NaF, 40 mg; $Al_2(SO_4)_3K_2SO_4 \cdot 24\ H_2O$, 8 mg; KI, 3.2 mg; $CoCO_3$, 3.2 mg; $Na_2SeO_3 \cdot 5\ H_2O$, 0.4 mg]; and oil mixture, 50 (50% "primor" rapeseed oil, 20% sunflower oil and 30% hydrogenated palm oil). The control diet was provided *ad libitum*. After five weeks, the animals were divided into three groups. One group (control) continued to receive the control diet. The other two groups were fed a similar diet in which the oil mixture was replaced either by one containing FO (35% sunflower oil, 50% hydrogenated palm oil and 15% salmon oil) or containing AA (45% "primor" rapeseed oil, 14% sunflower oil, 35% hydrogenated palm oil and 6% AA concentrate). Table 1 shows that the three oil mixtures had a similar n-6/n-3 FA ratio close to 6. Only the FO and AA oil mixtures contained FA whose carbon numbers were greater than 18, so that the FO mixture was characterized by the presence of n-3 PUFA (20:5 and 22:6) and the AA mixture by the presence of n-6 PUFA (20:4). Animals had free access to tap water. Food intake was limited to 20 g per day (70% of *ad libitum*) to prevent hyperphagia of obese Zucker rats. After seven weeks, the rats were starved for 16 h and exsanguinated (between 0700 and 0800 h) under diethyl ether anesthesia, and the livers were quickly removed (see below).

Preparation of a percoll-purified mitochondrial fraction (PPMF). The liver was transferred to aqueous medium containing 0.25 M sucrose, 10 mM *tris*(hydroxymethyl)aminomethane (Tris)/HCl, pH 7.4, and 1 mM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), and kept at 4°C. The liver was cut into small pieces, rinsed several times, blotted with paper and weighed. The tissue was then homogenized in 10 vol of sucrose medium containing, in addition, 1% albumin (24,25) with three strokes of a loose-fitting Teflon pestle at 300 rpm in an ice-cooled Potter–Elvehjem homogenizer (Bioblock, Strasbourg, France). The homogenate was centrifuged at 2,000 × *g* for 2 min (at 3°C in a JA-20 rotor, J-21 Beckman centrifuge; Beckman Instruments, Fullerton, CA), and the first crude mitochondrial pellet (M1) was obtained by centrifugation of the supernatant at 13,000 × *g* for 2 min. The procedure was repeated by homogenization of the low-speed pellet to generate a second crude mitochondrial pellet (M2). A third fraction (M3) was generated from the second low-speed pellet in an identical manner. The three crude mitochondrial pellets were resuspended in the above medium without albumin, pooled and centrifuged at 13,000 × *g* for 2 min to generate the crude mitochondrial fraction (CMF). The CMF was suspended in a mixture of 0.25 M sucrose medium containing 10 mM Tris/HCl buffer, pH 7.4, 1 mM EGTA, and Percoll (31% by volume of the final mixture) as described by Zammit *et al.* (26). This mitochondrial suspension (20 mL) was then layered on top of 20 mL of a Percoll mixture prepared as above, but containing a higher concentration of sucrose (0.3 instead of 0.25 M) using 40 mL centrifuge tubes. After centrifugation at 30,000 x *g* for 20 min in a JA-20 rotor (J-21 Beckman), the mitochondria, which sediment as a fluffy layer, were withdrawn, diluted in buffered 0.25 M sucrose, washed free of percoll by centrifugation at 3,500 × *g* for 10 min, and stored in buffered 0.3 M sucrose as the PPMF.

Isolation of mitochondrial OM. Mitochondrial OM were prepared according to the modified procedure of Parsons *et al.* (27). The PPMF was centrifuged at 13,000 × *g*. The resulting pellet was suspended in hypoosmotic medium containing 20 mM KH_2PO_4, pH 7.4, and 0.02% FA-free albumin (240 to 320 mL) using two mild hand-driven strokes of a Dounce B homogenizer (Bioblock). After 20 min on ice, an amount of ATP (mg) corresponding to 1:5 of the mitochondrial protein content, previously estimated by ultraviolet assay (28), was added and the incubation was left at 0°C for a further 5 min. OM were separated from the inner membrane–matrix fraction by sedimentation at 30,000 × *g* for 8 min, followed by resuspension and centrifugation at 3,500 × *g* for 8 min. The crude OM were recovered from the supernatant by centrifugation at 30,000 × *g* for 8 min. Further purification was achieved on a discontinuous sucrose gradient at 80,000 × *g* for 3 h. OM were collected at the interface of the layers of density 1.1175 and 1.1390, diluted in 3 vol of 20 mM KH_2PO_4, pH 7.4, concentrated by centrifugation at 150,000 × *g* for 30 min, and resuspended in buffered 0.25 M sucrose as the mitochondrial OM fraction.

Microsomal fraction. The supernatant of the first crude mitochondrial pellet (M1) was centrifuged at

18,000 × *g* for 20 min at 3°C to yield a pellet containing mitochondria, peroxisomes and part of the microsomes (not shown). The supernatant was centrifuged at 104,000 × *g* for 40 min at 3°C using a Ti-60 rotor (L8-55 Beckman), and the resulting pellet was suspended in sucrose medium, layered on top of 1.2 M sucrose medium and sedimented as above to remove cytosolic proteins and albumin originating from the homogenization step. The microsomal pellet was easily separated from the lower translucent layer of glycogen by gentle shaking, then stored in buffered 0.25 M sucrose as the microsomal fraction.

Enzyme assays. The presence of mitochondrial, peroxisomal and microsomal organelles was estimated by the activities of monoamine oxidase (29), urate oxidase (30) and aryl-ester hydrolase (31), respectively. The peroxisomal fatty acid-oxidizing system (PFAOS) was determined by CN-insensitive palmitoyl-CoA dependent NAD^+ reduction (32,33) in the presence of 75 μM palmitoyl-CoA. Measurements of CPT I activity were carried out at 30°C according to Bremer (34) with slight modifications (35). Because microsomes have been shown to display carnitine acyltransferase activity (36), the PPMF which were least contaminated by microsomes (Table 4), were preferred over other mitochondrial fractions for CPT I measurements. The sensitivity of CPT I to malonyl-CoA inhibition was estimated by incubating mitochondrial proteins with palmitoyl-CoA (40 μM) and the indicated concentrations of malonyl-CoA for 2 min before addition of L-[^{3}H]carnitine (400 μM, 1 Ci/mol). After 4 min, the reaction was stopped by addition of acid, and the acyl-[^{3}H]carnitine was extracted with *n*-butanol (37). Radioactivity was quantitated using Picofluor 15 (Packard Instrument Co., Meriden, CT) in a Packard 300 C scintillation counter.

Lipid analysis. Total lipids of liver and subcellular fractions were extracted by the procedure of Folch *et al.* (38) and saponified with 10% KOH in methanol (wt/vol) at 100°C for 1 h. Free FA with heptadecanoic acid added as an internal standard were extracted with hexane from the acidified medium and treated, after evaporation of hexane, with H_2SO_4/methanol (5:95, vol/vol) at 100°C for 3 h. The methyl esters were extracted with hexane and analyzed by capillary gas–liquid chromatography (CGLC) on a model 419 Becker–Packard (Downer's Grove, IL) apparatus fitted with a laboratory made 30 m × 0.3 mm i.d. glass capillary column coated with carbowax 20M. CGLC peaks were identified based on their retention time relative to methyl heptadecanoate. Peak areas were measured using a Delsi model Enica 21 computing integrator (Delsi Instruments, Suresnes, France). The separation of PL classes was achieved by high-performance liquid chromatography (39) on a Varian apparatus (Walnut Creek, LA), using a Hibar Merck 250-4 column (Merck) packed with Lichrosorb Si-60 (particle diameter, 5 μm). Mass was estimated by adding a known amount of heptadecanoic acid to each fraction collected. PL FA were directly transesterified with methanol/H_2SO_4 after drying the samples, and were analyzed by CGLC (see above). For measuring TAG, total liver lipids were extracted in the presence of 1,2,3-triheptadecanoylglycerol as an internal standard. TAG were separated by thin-layer chromatography, then saponified, and the FA were methylated and analyzed by CGLC as described above. The cholesterol content of the lipid extracts was measured by CGLC (40) on a model 419 Becker-Packard chromatograph fitted with a 10 m × 0.3 mm i.d. glass capillary column coated with SE-30. Epicoprostanol and α-cholestane were added as internal standards.

Protein. Rapid protein estimations were obtained by spectrophotometry (A280) (28) just before starting the incubations; values were later more accurately determined by the bicinchoninic acid procedure (41).

Statistics. Results are expressed as means ± SE. Statistical comparisons of differences between groups were analyzed by one-way analysis of variance and Fisher's test.

RESULTS AND DISCUSSION

Body and liver weights. An interesting observation that was made in this study is that n-3 PUFA of FO, when present at a level of only 0.2% of the diet (n-3 unsaturated FA constitute 4.1% of the FO mixture; see Table 1), are able to reduce liver weight and liver TAG content of obese Zucker rats (Table 2) whose lipogenesis and esterification pathways are known to be very active (20,21). Values obtained for the rats fed the diet enriched in AA did not differ from those of controls.

TABLE 1

Fatty Acid Composition[a] of the Oil Mixtures Present in the Three Diets[b]

Fatty acids	FO mixture	Control mixture	AA mixture
14:0	1.7	0.8	0.6
16:0	27.2	16.5	16.8
18:0	6.0	4.4	3.9
20:0	0.3	0.4	0.4
22:0	0.4	0.3	0.3
Total saturated	35.6	22.4	22.0
16:1n-7	1.5	—	—
18:1n-9	30.9	43.9	44.7
18:1n-7	1.0	3.2	1.8
20:1n-9	1.1	0.6	0.7
22:1n-11	0.5	—	—
Total monounsaturated	35.0	47.7	47.2
18:2n-6	25.3	25.6	21.5
20:4n-6	—	—	4.2
Total n-6 unsaturated	25.3	25.6	25.7
18:3n-3	0.2	4.3	3.9
18:4n-3	0.3	—	—
20:5n-3	1.6	—	—
22:5n-3	0.4	—	0.4
22:6n-3	1.6	—	—
Total n-3 unsaturated	4.1	4.3	4.3
n-6/n-3 ratio	6.2	6.0	6.0

[a]The composition of fatty acids is given as weight percentage of total fatty acids in the oil mixtures added to the three diets.
[b]FO (fish oil) refers to the oil mixture containing salmon oil. Arachidonic acid (AA) refers to the oil mixture enriched in AA.

TABLE 2

Effects of Diets[a] Enriched in n-3 and n-6 Fatty Acids on Body and Liver Weights[b] and on Lipid Content[b,c] of Liver

	FO	Control	AA
Body weight			
(g)	544 ± 18	550 ± 16	581 ± 30
Liver weight			
(g)	18.5 ± 1.0[d]	21.5 ± 0.9[e]	22.7 ± 1.3[e]
(% of body weight)	3.40 ± 0.15[d]	3.88 ± 0.10[e]	3.89 ±
Liver content in			
Total lipids	122 ± 15[d]	162 ± 19[e]	171 ± 12[e]
Triacylglycerols	77 ± 8[d]	101 ± 12[e]	103 ± 9[e]

[a]FO and AA refer to the diets enriched in n-3 fatty acids with FO and in n-6 fatty acids with the AA concentrate, respectively. See Table 1 for abbreviations.
[b]Results are means ± SE (n = 5). Values in a row with different superscript letters (d,e) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons.
[c]The contents in total lipids and in triacylglycerols are expressed as mg of fatty acids per gram of liver.

TABLE 3

Effects of Diets[a] on Mitochondrial and Peroxisomal Activities[b] in the Liver of Obese Zucker Rats

	FO	Control	AA
Monoamine oxidase			
μmol/min/g liver	0.78 ± 0.05[d]	0.60 ± 0.04[e]	0.48 ± 0.02[f]
μmol/min/total liver	14.5 ± 0.8[d]	12.8 ± 0.9[d]	10.8 ± 0.5[e]
Urate oxidase			
μmol/min/g liver	1.96 ± 0.08[d]	1.60 ± 0.07[e]	0.75 ± 0.04[f]
μmol/min/total liver	36.3 ± 1.6[d]	34.4 ± 1.7[d]	17.0 ± 1.0[e]
PFAOS[c]			
μmol/min/g liver	0.47 ± 0.02[d]	0.39 ± 0.02[e]	0.13 ± 0.02[f]
μmol/min/total liver	8.79 ± 0.37[d]	8.45 ± 0.38[d]	2.86 ±

[a]FO and AA as defined in Tables 1 and 2.
[b]Results are means ± SE (n = 5). Values in a row with different superscript letters (d,e,f) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons.
[c]PFAOS, peroxisomal fatty acid-oxidizing system.

Enzyme activities in liver tissue and mitochondrial fractions. In Table 3, the activities of mitochondrial and peroxisomal enzymes per unit volume of homogenate (corresponding to 1 g liver) are given. The FO diet caused a significant enhancement of mitochondrial (monoamine oxidase) and peroxisomal (urate oxidase and PFAOS) activities per gram liver, but not on a total liver basis (compared to control diet). As the monoamine oxidase specific activity did not differ to any great extent in the purest of the mitochondrial fractions (Table 4), the mitochondrial content should be very similar per whole liver both in FO and control rats. In addition, according to data showing that FO induces an increase in peroxisomal activities when present at a level greater than 15% in the diet (13,14), the results we obtained using a low level of FO (0.75%, because FO represents

TABLE 4

Specific Activities[a] of Enzymes Present in Mitochondrial and Microsomal Fractions Isolated from Liver of Obese Zucker Rats Fed Diets[b] Enriched in PUFA

	FO	Control	AA
Monoamine oxidase			
Crude mitochondrial fraction	10.8 ± 0.4[c]	8.6 ± 0.3[d]	10.4 ± 0.5[c]
Percoll-purified fraction	12.6 ± 0.4[c]	10.3 ± 0.3[d]	12.3 ± 0.4[c]
Outer membrane fraction	211 ± 7[c]	177 ± 6[d]	187 ± 7[d]
Microsomal fraction	0	0	0
Urate oxidase			
Crude mitochondrial fraction	40 ± 3	38 ± 5	39 ± 4
Percoll-purified fraction	4 ± 1	3 ± 1	3 ± 1
Outer membrane fraction	0	0	0
Microsomal fraction	6 ± 2	5 ± 1	4 ± 1
Aryl-ester hydrolase			
Crude mitochondrial fraction	517 ± 13	503 ± 11	499 ± 13
Percoll-purified fraction	132 ± 4[c]	104 ± 5[d]	146 ± 4[e]
Outer membrane fraction	325 ± 8[c]	367 ± 9[d]	368 ± 7[d]
Microsomal fraction	2940 ± 120[c]	2320 ± 100[d]	2850 ± 110[c]
Microsomal contamination			
In crude mitochondrial fraction	17.6	21.6	17.5
in percoll-purified fraction	4.5	4.5	5.1
In outer membrane fraction	11.1	15.8	12.9

[a]Enzyme activities are given in nmoles/min/mg protein. Values are means ± SE (n = 5). Values in a row with different superscript letters (c,d,e) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons. Microsomal contamination is expressed as mg of microsomal protein per 100 mg of protein of the mitochondrial fraction studied and was calculated as follows: the specific activity of aryl-ester hydrolase in the mitochondrial fraction studied was divided by that in the microsomal fraction of the same liver, and multiplied by 100.
[b]FO and AA as defined in Table 1.

15% of the total oil mixture; see Materials and Methods section) failed to show any enhancement of PFAOS activity per total liver. Consequently peroxisomes were not likely to lower hepatic TAG levels by increasing FA oxidation, as it has been suggested for diets containing FO at higher levels (12,13,42) or only EPA (43). By contrast, the AA diet diminished mitochondrial and peroxisomal enzymatic activities expressed either per gram or total liver (Table 3). These data and the fact that the monoamine oxidase specific activity was similar in the purest of the mitochondrial fractions from AA and control rats (Table 4) would suggest a decrease in mitochondrial mass per gram and total liver of AA rats. Hence, the decreased mitochondrial mass and the lower PFAOS activity should reduce the degree of FA oxidation per total liver in rats fed the AA diet.

Peroxisome and microsome contamination in mitochondrial fractions (Table 4). As monoamine oxidase specific activity was measured in mitochondrial fractions (whole mitochondria or OM) that were not totally pure, it was necessary to assess the amount of the main contaminating organelles that would be likely to influence the specific activity of the mitochondrial enzyme (seen above) and membrane lipid composition. Since urate oxidase specific activities (Table 4) were similar in the liver of rats maintained on any of the three diets (in each fraction) and since urate oxidase specific activity has been reported to be very high in pure peroxisomes, cross-contamination seems unlikely to affect the comparisons between the diets to any extent. For microsomes contaminating the mitochondrial fractions, the specific activity of aryl-ester hydrolase found in mitochondrial and microsomal fractions enabled us to calculate that the crude mitochondrial fractions contained similar amounts of microsomes (between 17 and 21% on a protein basis). In percoll-purified fractions, the values were much lower and corresponded to about 5% of microsomal proteins. Hence, the similar level of contamination of each fraction (for the three diets) by nonmitochondrial organelles would explain the similarity of monoamine oxidase specific activities obtained for the three fractions corresponding to the three diets. In OM fractions, which were devoid of urate oxidase activity, aryl-ester hydrolase activity per mg protein was found to be even greater than in percoll-purified fractions. This microsomal contamination, which represented 11.1 and 15.8% of total protein with the FO and control diets, respectively, could greatly influence the true FA composition and cholesterol content of OM. Attempts to effect a more thorough removal of the microsomes failed, and microsomal membranes already present in percoll-purified fractions seemed to comigrate with OM in the sucrose gradient. The data suggest that microsomal membranes are tightly bound to OM, as has already been suggested (44–47). This also implies that the degree of contamination has to be taken into account when determining the lipid composition of OM. Very long-chain FA are poor substrates for mitochondrial FA oxidation (48) and probably are accumulated as CoA derivatives (49) even when present at low levels in the diet. Since a large part of cellular CoA may be sequestered as long-chain acyl-CoA esters, one role of the aryl-ester hydrolase of the endoplasmic reticulum has been suggested to be to limit the content in acyl-CoA (50). In this respect, the higher specific activity of the aryl-ester hydrolase found in microsomal fractions of rats fed the FO and AA diets would constitute a response to a permanently higher ester content. It could then be expected that accumulated very long-chain acyl-CoA would exert inhibitory effects on enzyme activities related to FA synthesis, as has already been observed in whole animals (51) and in isolated hepatocytes (52).

FA composition of total mitochondrial lipids. It is well documented that dietary FA directly influence the composition of biological membranes and corresponding enzymatic activities (53). Measurements made on whole mitochondria rather reflected the composition of the inner membranes probably because of their extensive surface area. In mitochondria (Table 5), the percentages of 20:4n-6 were 22.2 and 25.3%, while those of 22:6n-3 were 16.9 and 13.1% in FO and control rats, respectively. These differences may not be of great importance in respect to some inner membrane-bound activities since rats fed a diet deficient in α-linolenic acid, 18:3n-3, did not display any change in the activity of the respiratory chain and of F_1F_0-ATPase in spite of a low percentage of 22:6n-3 and a higher percentage of 20:4n-6 (54). However, it is clear that the FO diet favored the accumulation of DHA into mitochondrial membranes in spite of the fact that DHA represented only 1.6% of the FA in the FO mixture. In this respect, Yamaoka *et al.* (55) reported that a diet containing 20% of sardine oil (compared to corn oil) enhanced the proportion of DHA up to two to three times in the various PL of liver mitochondrial membranes. With the FO diet, accumulation of 20:5n-3 cannot be observed (Table 5), but this dietary FA may have been derived through desaturation–elongation reactions to cause the significant enhancement of the level of DHA as shown above. Supplementation of the diet with AA was not accompanied by a corresponding enrichment of mitochondrial lipids with this FA.

TABLE 5

Fatty Acid Composition[a] of Total Lipids of Mitochondrial Fractions[b] Isolated from Liver of Obese Zucker Rats Fed Diets[c] Enriched in Polyunsaturated Fatty Acids

Fatty acids	FO	Control	AA
16:0	16.2 ± 1.8	18.0 ± 1.6	17.1 ± 2.0
16:1	—	—	0.2 ± 0.1
18:0	14.7 ± 0.6[d]	16.0 ± 0.5[e]	15.8 ±
18:1	13.1 ± 0.4[d]	13.0 ± 0.4[d]	11.0 ± 0.3[e]
18:2n-6	10.3 ± 0.3	7.3 ± 0.3[e]	8.3 ± 0.4[e]
20:4n-6	22.2 ± 2.0	25.3 ± 1.9	24.7 ± 2.1
20:5n-3	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
22:5n-3	0.4 ± 0.2	0.8 ± 0.2	1.1 ± 0.3
22:6n-3	16.9 ± 1.2[d]	13.1 ± 0.7[e]	11.7 ± 0.5[f]

[a]Values are means ± SE (n = 5) and are given in weight percentage relative to total fatty acid mass. Values in a row with different superscript letters (d,e,f) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons.
[b]Percoll-purified mitochondrial fractions.
[c]FO and AA as defined in Table 2.

This would provide evidence that the requirement for AA was easily met through synthesis starting from 18:2n-6 in animals fed the other two diets.

FA composition of PL in OM fractions. Due to the fact that CPT I is located in the mitochondrial OM and that its activity seems to be influenced by the surrounding PL (56), the FA composition of the major PL classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was determined. Independent of the diet, PC and PE were shown to represent about 57 and 29% of total PL, respectively (Table 6). The FA compositions for both classes of PL appeared qualitatively similar to those obtained for total mitochondria. The FO diet gave rise to the highest percentage of DHA both in PC and PE, while the AA diet led to the lowest percentage of DHA and only to a slightly higher level of 20:4n-6. None of the differences between diets exceeded 6%. In general, it appears that dietary DHA and 20:4n-6 directly enhanced their own presence in OM and conversely diminished each other's level. Since some microsomal membranes were bound to OM membranes, the FA compositions were also determined for PC and PE of the microsomal fractions (Table 7). The percentages of PC and PE in total PL were similar for each, independent of the diet, i.e., they were 63 and 19%, respectively. The FA compositions showed great similarities with those found for the OM fractions. Therefore, the FA composition of pure OM should be very similar to that of OM fractions containing microsomes.

Cholesterol content in mitochondrial and microsomal fractions. The cholesterol content was found to be highest in microsomal fractions and lowest in percoll-purified mitochondrial fractions (Table 8). Cholesterol levels were appreciable in crude mitochondrial fractions and even higher in OM fractions. Since the amount of contaminating microsomes in these fractions was known, the cholesterol content in these fractions devoid of microsomes could be calculated. The corrected values were found to be extremely low and were similar for the three diets in the percoll-purified fractions (about 0.35 µg per mg protein). The OM fractions had the highest cholesterol content (about 11.8 µg per mg protein), so that all mitochondrial cholesterol must essentially be localized in the OM.

CPT I activity and its sensitivity to malonyl-CoA. The specific activity of CPT I in the absence of malonyl-CoA was found to be highest in mitochondria from rats on the control diet and lowest for rats on the AA diet (Fig. 1) The FO diet was shown to reduce the sensitivity of CPT I to malonyl-CoA, in contrast to the AA diet, which clearly enhanced this sensitivity (Fig. 2). Kolodziej and Zammit (57) showed that OM, when exposed to membrane fluidizers such as benzyl alcohol, showed a slightly enhanced CPT I activity and a decreased sensitivity to malonyl-CoA. Therefore, alterations in lipid composition may have an effect on CPT I activity, as has already been shown by Brady *et al.* (58). The similar cholesterol content found in OM of FO and control rats cannot account for any differential effects of this molecule, which is considered to rigidify membrane organization (53). However, the proportion of DHA was always observed to be 4 to 5% higher in PC and PE of OM isolated from rats fed the FO diet. The lower specific activity of CPT I found with the FO diet by comparison to the con-

TABLE 6

Fatty Acid Compositions[a] of PC and PE of Mitochondrial Outer Membranes[b] Isolated from Liver of Obese Zucker Rats Fed Diets[c] Enriched in Polyunsaturated Fatty Acids

	PC			PE		
Fatty acids	FO	Control	AA	FO	Control	AA
16:0	22.2 ± 1.9	22.6 ± 1.5	22.3 ± 2.0	18.4 ± 1.6	18.0 ± 1.4	17.9 ± 1.5
16:1	1.0 ± 0.1	1.9 ± 0.2^{e}	2.0 ± 0.2^{e}	—	—	0.9 ± 0.2
18:0	17.5 ± 1.7	17.9 ± 1.8	18.6 ± 1.5	20.8 ± 1.9	20.4 ± 2.0	20.8 ± 1.8
18:1	8.2 ± 1.3	9.7 ± 1.2	9.8 ± 1.4	4.3 ± 1.0^{d}	5.9 ± 0.9^{d,e}	7.2 ± 1.0^{e}
18:2n-6	4.2 ± 0.4^{d}	3.2 ± 0.3^{e}	2.5 ± 0.3^{f}	1.4 ± 0.2	1.4 ± 0.4	1.4 ± 0.3
20:4n-6	26.2 ± 2.3^{d}	31.1 ± 2.1^{e}	33.7 ± 2.2^{e}	20.6 ± 1.2^{d}	23.6 ± 1.5^{e}	26.6 ± 1.6^{f}
20:5n-3	0.6 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	1.6 ± 0.2^{d}	0.5 ± 0.1^{e}	0.4 ± 0.1^{e}
22:5n-3	0.5 ± 0.1	0.76 ± 0.20	0.59 ± 0.10	1.1 ± 0.2^{d}	1.96 ± 0.20^{e}	1.57 ± 0.30^{d,e}
22:6n-3	13.3 ± 1.7^{d}	9.4 ± 1.2^{e}	8.1 ± 1.1^{e}	29.0 ± 2.3^{d}	23.8 ± 2.0^{e}	20.1 ± 1.5^{f}
Total saturated	39.7 ± 1.8	40.5 ± 1.9	40.9 ± 2.0	39.5 ± 1.8	38.4 ± 1.9	38.7 ± 1.7
Total n-6	34.4 ± 2.1	35.4 ± 1.9	36.2 ± 2.0	22.9 ± 1.2^{d}	25.6 ± 1.5^{e}	29.6 ± 1.6^{f}
Total n-3	14.5 ± 1.7^{d}	10.4 ± 1.2^{e}	8.8 ± 1.2^{e}	31.7 ± 2.3^{d}	26.3 ± 2.0^{e}	22.0 ± 1.5^{f}
Total $\geq C_{20}$ unsaturated	40.6 ± 2.1	41.5 ± 2.0	42.5 ± 2.1	52.3 ± 2.3	49.9 ± 1.9	48.7 ± 1.7

[a]PC and PE were separated by high-performance liquid chromatography. As a percentage of total phospholipid mass, phosphatidylcholine (PC) constituted 56.8 ± 0.5, 57.8 ± 0.5, 57.0 ± 0.3, and phosphatidylethanolamine (PE) 29.5 ± 0.2, 29.5 ± 0.2, 29.2 ± 0.3 for FO, control and AA diets, respectively. The fatty acid compositions were analyzed by capillary gas–liquid chromatography. Values are means ± SE (n = 5) and are given in weight percentage relative to total fatty acids. Values in a row for PC or PE with different superscript letters (d,e,f) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons. The terms total saturated, total n-6, total n-3 and total unsaturated $\geq C_{20}$ refer to the sum of saturated, of n-6, of n-3 and of $\geq C_{20}$ unsaturated fatty acids, respectively.

[b]Membranes isolated from percoll-purified mitochondrial fractions.

[c]FO and AA as defined in Table 1.

TABLE 7

Fatty Acid Compositions[a] of PC and PE of Microsomal Fractions Isolated from Liver of Obese Zucker Rats Fed Diets[b] Enriched in Polyunsaturated Fatty Acids

	PC			PE		
Fatty acids	FO	Control	AA	FO	Control	AA
16:0	22.1 ± 1.5	23.8 ± 1.3	22.0 ± 1.3	17.2 ± 1.2[c]	17.9 ± 1.3[c]	22.8 ± 1.5[d]
16:1	1.4 ± 0.2	1.6 ± 0.2	1.8 ± 0.3	—	0.8 ± 0.2	—
18:0	18.2 ± 1.1	17.1 ± 1.2	17.1 ± 1.4	22.0 ± 1.9	20.5 ± 2.0	20.0 ± 1.8
18:1	8.7 ± 0.6[c]	10.1 ± 0.7[d]	14.2 ± 0.9[e]	4.0 ± 0.8[c]	7.2 ± 0.8[d]	7.2 ± 0.9[d]
18:2n-6	3.9 ± 0.6	3.0 ± 0.4	3.6 ± 0.4	1.5 ± 0.6	1.7 ± 0.4	1.8 ± 0.5
20:4n-6	26.1 ± 1.4[c]	29.0 ± 1.3[d]	28.8 ± 1.9[c,d]	22.4 ± 1.8[c]	23.5 ± 1.9[c]	27.3 ± 2.0[d]
20:5n-3	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	1.0 ± 0.3
22:5n-3	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.3	1.3 ± 0.4	2.1 ± 0.4	1.6 ± 0.3
22:6n-3	13.9 ± 1.3[c]	9.1 ± 1.1[d]	6.7 ± 0.7[e]	27.4 ± 2.2[c]	20.8 ± 2.0[d]	20.2 ± 2.2[d]
Total saturated	40.3 ± 1.5	40.9 ± 1.3	39.1 ± 1.4	39.2 ± 1.8	38.4 ± 2.0	42.8 ± 1.9
Total n-6	30.0 ± 1.4	32.0 ± 1.4	32.4 ± 1.8	23.9 ± 1.7[c]	25.2 ± 1.8[c]	29.1 ± 1.9[d]
Total n-3	15.2 ± 1.3[c]	10.0 ± 1.2[d]	7.5 ± 0.7[e]	29.3 ± 2.1[c]	23.2 ± 1.9[d]	22.7 ± 2.2[d]
Total $\geq C_{20}$ unsaturated	41.3 ± 1.6[c]	47.1 ± 1.4[d]	36.3 ± 2.0[e]	51.7 ± 2.5	46.7 ± 2.0	50.1 ± 2.2

[a]PC and PE were separated by high-performance liquid chromatography. As a percentage of total phospholipid mass, PC constituted 63.2 ± 0.5, 62.2 ± 0.7, 64.0 ± 0.8, and PE 19.0 ± 0.3, 19.4 ± 0.3, 18.6 ± 0.4 for FO, control, and AA diets, respectively. The fatty acid compositions were analyzed by capillary gas–liquid chromatography. Values are means ± SE (n = 5) and are given in weight percentage relative to total fatty acids. Values in a row for PC or PE with different superscript letters (c,d,e) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons. The terms total saturated, total n-6, total n-3 and total unsaturated $\geq C_{20}$ refer to the sum of saturated, of n-6, of n-3 and of $\geq C_{20}$ unsaturated fatty acids, respectively.
[b]FO and AA as defined in Table 1. See Tables 2 and 6 for other abbreviations.

trol diet seems to be in disagreement with previous data that had shown no difference in enzyme activities (11) or, oppositely, had shown a more enhanced CPT I specific activity in rats (14) and mice (16) when fed diets containing higher levels of FO. The major differences with respect to our studies were the very low level of FO that we added to the diet and the obesity of Zucker rats we used. The slight difference in FA composition particularly with regard to DHA, between animals fed FO and control diets would be sufficient to alter the environment of the CPT I protein in the OM and to reduce the efficiency of the catalytic site. However, the complexity of characteristics of the enzyme (59), its differential susceptibility to detergents (60), and the additional possibility that the lower activity with the FO diet was due to a change in enzyme mass, makes it difficult to formulate a conclusive explanation at this stage. The most interesting observation concerns the lower sensitivity of CPT I to malonyl-CoA inhibition with FO rats. This is considered more important in the control of FA oxidation than the enzymatic activity itself, at least under some conditions (see, for example, Ref. 61). With the FO diet, the lower sensitivity becomes an even more preponderant factor because of the usually elevated amount of malonyl-CoA in the liver of obese Zucker rats (62,63). Moreover, as mentioned above, some PUFA of FO have been shown to inhibit FA synthesis and related enzyme activities such as acetyl-CoA carboxylase. A lower sensi-

TABLE 8

Effects of Diets[a] on Total Cholesterol Content[b] in Mitochondrial and Microsomal Fractions of Obese Zucker Rat Liver

	FO	Control	AA
Crude mitochondrial fraction	8.20 ± 0.25	8.90 ± 0.28	8.40 ± 0.26
Percoll-purified fraction	2.40 ± 0.16[c,d]	2.20 ± 0.15[c]	2.50 ± 0.16[d]
Outer membrane fraction	15.7 ± 0.4	16.2 ± 0.5	16.5 ± 0.5
Microsomal fraction	46 ± 2	41 ± 2	43 ± 2
Values after correction from microsomal cholesterol contamination			
Percoll-purified fraction	0.35 ± 0.02	0.37 ± 0.02	0.32 ± 0.02
Outer membrane fraction	11.9 ± 0.4	11.5 ± 0.3	11.8 ± 0.3

[a]FO and AA as defined in Table 1.
[b]Results are expressed as μg of total cholesterol per mg protein and are means ± SE (n = 5). The values in a row with different superscript letters (c,d) are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons. Microsomal cholesterol contamination was calculated by multiplying the amount (mg) of microsomal protein contaminating one mg of a mitochondrial fraction (see Table 4) by the cholesterol content of one mg protein of the microsomal fraction isolated from the same liver.

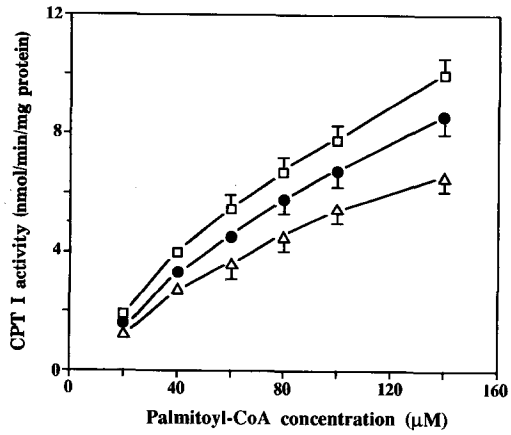

FIG. 1. Effect of increasing palmitoyl-CoA concentrations on the activity of carnitine palmitoyl-transferase I (CPT I) in percoll-purified mitochondrial fractions isolated from liver of obese Zucker rats fed the control diet (□), the fish oil diet (●) or the arachidonic acid diet (△). Assays were performed as described in the Materials and Methods section using 200 μg of mitochondrial protein in the presence of palmitoyl-CoA and L-[^{3}H]carnitine. The radioactivity of the butanolic extract corresponded to carnitine-bound compounds. Results are expressed as nmoles of palmitoyl-carnitine formed per min per mg protein. T-bars show SE (n = 5). Values obtained for 80 to 140 μM palmitoyl-CoA between the groups are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons.

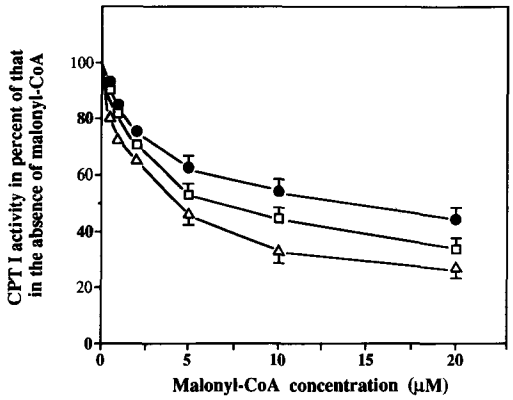

FIG. 2. Inhibitory effect of malonyl-CoA on the activity of carnitine palmitoyl-transferase I (CPT I) in percoll-purified mitochondrial fractions isolated from liver of obese Zucker rats fed the control diet (□), the fish oil (FO) diet (●) or the arachidonic acid (AA) diet (△). Mitochondria were kept 2 min in the presence of 40 μM palmitoyl-CoA and malonyl-CoA before adding labeled L-carnitine. Results are given as percentage of the CPT I activity obtained in the absence of malonyl-CoA. A 50% inhibition by malonyl-CoA was seen at the concentration of 6 μM with the control diet, at 16 μM with the FO diet, and at 4.5 μM with the AA diet. T-bars show SE (n = 5). Values obtained for 10 to 20 μM malonyl-CoA between the groups are significantly different at $P < 0.05$, as determined by analysis of variance and Fisher's test comparisons. See Figure 1 for abbreviation.

tivity to malonyl-CoA associated with a lesser malonyl-CoA synthesis would give rise to a proportionally higher activity of CPT I and a higher rate of FA oxidation.

Results obtained with the AA diet strongly differed from those obtained with the FO diet. AA was present at a level of only 0.2% of the diet (20:4n-6 represented 4.2% of the total AA oil mixture; see Table 1). In contrast to what has been seen for FO rats, liver weights and TAG contents were similar in AA and control rats (Table 2). In rats fed the AA diet, we saw a decrease in PFAOS activity and in mitochondrial protein mass per total liver. Moreover, CPT I specific activity was found to be lowest and its sensitivity to malonyl-CoA inhibition highest from rats on the three diets. A lower rate of FA oxidation by peroxisomes and mitochondria should have favored TAG formation when compared to the control group. At this stage, it is difficult to relate the slightly higher content in 20:4n-6 and/or the lower DHA content of OM PL to the altered characteristics of CPT I enzyme from AA rats compared to controls. However, since rats fed the control and FO diets were capable of producing 20:4n-6 from shorter precursors, it appears that AA was supplied in excess to the cell with the AA diet. As dietary AA (or its acyl-CoA) has been shown to inhibit FA synthetase and acetyl-CoA carboxylase (64,65), less malonyl-CoA synthesis would favor CPT I activity in the intact cell. In addition, the steady intake of AA, which is the major membrane PUFA, should save a large portion of the energy usually required for synthesis. The lower mitochondrial mass per total liver in AA rats might correspond, at least in part, to an adaptive response to less need for energy. In this respect, the amount of AA might represent, by itself and/or through its derivatives, an effective signal to induce metabolic change allowing, in particular, the regulation of mitochondriogenesis.

The major finding of this study was that we could show that the activities that relate to FA oxidation were altered in the liver of obese Zucker rat when the diets contained very low levels of n-3 and n-6 PUFA. The altered composition of mitochondrial OM in n-3 and n-6 PUFA, but not in cholesterol, seems to be sufficient to influence, *via* the kinetic characteristics of CPT I, the pathway of FA oxidation.

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Dietary Linoleic, α-Linolenic and Oleic Acids Are Oxidized at Similar Rates in Rats Fed a Diet Containing These Acids in Equal Proportions

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The objective of this study was to examine whether whole body oxidation rates of dietary linoleic, α-linolenic and oleic acids differ when the acids are provided in identical quantities. Male rats were fed for 10 wk a 15% fat (w/w) diet containing equal amounts of linoleic, α-linolenic and oleic acids (22.7, 23.0 and 23.2% of total fatty acids, respectively). At week 10, after overnight fasting, rats were intragastrically administered 20 μCi of either [1-^{14}C]-labelled linoleic, α-linolenic or oleic acid in a 200-μL bolus of oil containing equal quantities of each fatty acid. The appearance of $^{14}CO_2$ in expired air was then monitored hourly for 12 h for each animal. A preliminary study had shown that growth and food consumption patterns in animals consuming the oil containing equal quantities of each of the fatty acids paralleled the patterns of animals that were self-selecting among separate diets, each of which contained one of the component oils. The appearance of ^{14}C, expressed as percent dose administered, peaked at 2–3 h post-dose for ^{14}C-labelled linoleic (5.28 ± 0.37%/h), α-linolenic (6.92 ± 0.51%/h) and oleic (5.98 ± 0.44%/h) acids. Statistically these values were not significantly different. Cumulative $^{14}CO_2$ excretion rates over 12 h were also similar for linoleic (27.2 ± 0.9%), α-linolenic (26.8 ± 1.2%) and oleic (25.9 ± 1.2%) acids. The results suggest that the rat's capacity to oxidize 18-carbon unsaturated fatty acids is not affected by fatty acid unsaturation when these fatty acids are provided at equal dietary levels.

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A number of previous studies have indicated that disposal of dietary fat for energy rather than storage depends on the long-chain fatty acid composition of the fat (1–11). Respiratory gas exchange data obtained in animal experiments suggested that greater peroxisomal oxidation (1) and oxygen consumption (2) occurs with consumption of fats rich in polyunsaturated fatty acids (PUFA) compared with fats containing monounsaturated (MUFA) or saturated fatty acids (SAFA). Similarly, energy intake/balance experiments have generally shown that consumption of n-3 PUFA results in lesser weight and energy gains when compared with fats containing other fatty acids (3–5).

Discrimination between various fatty acids in the oxidation process has also been suggested from animal and human experiments with tracer-labelled fatty acids (6–11). In rats fed normal chow diets and given ^{14}C-labelled long-chain fatty acids orally, differences between oxidation rates for α-linolenic acid, oleic acid and linoleic acid were seen over the first 8 h of a 24 h study, with α-linolenic acid being converted to carbon dioxide most rapidly, followed by oleic acid and linoleic acid (6). Arachidonic and γ-linolenic acid exhibited the lowest rates of oxidation. Other studies with labelled fatty acids suggested a similar trend of PUFA toward oxidation rather than retention within body pools (7–10). In humans, expired $^{13}CO_2$ from labelled oleic acid after oral administration appeared more rapidly than from labelled linoleic or stearic acids (11). The general consensus that emerged from these studies is that the blend of fatty acids consumed, which depends on the type of dietary fat or oil selected, can modify an individual's susceptibility to long-term body weight gain.

However, limitations exist in the interpretation of the results from previous experiments when one compares tracer appearance rates in expired CO_2 derived from labelled dietary fatty acids. A major difficulty has often been the vastly different quantity of specific fatty acids consumed. For instance, tracer fatty acids were typically added directly to dietary olive oil (6), or lard and corn oil followed by fasting (7), or were transesterified directly with soybean (8), olive (9) or corn oil (10). Also, tracer fatty acids were added directly to chow diets. Such diets contain, of course, widely varying pools of specific fatty acids. Thus, the quantities of tracee (i.e., the unlabelled fatty acid pool with which the tracer fatty acid became associated varied between experiments and fatty acids tested. Differences in the tracer/tracee ratio imply that it may be erroneous to conclude preferential fatty acid oxidation on the basis of a greater proportion of label excreted, if the tracer was mixed with a fatty acid pool of different size. Moreover, endogenous pools of specific fatty acids which are targeted for oxidation will likely be depleted or expanded in response to the exogenous fatty acid blend delivered by feeding the diet. For these reasons, interpretation of results from many of the earlier tracer studies has remained difficult. Various approaches aimed at controlling the tracer/tracee ratio for specific fatty acids at the time of label administration have previously been used (12); however, animals previously have not been prefed a diet with which internal body pool sizes could be standardized.

To permit the study of the oxidation capacity for specific dietary fatty acids under identical conditions, an approach was chosen here in which equivalent quantities of three important dietary fatty acids, differing in unsaturation, were provided during both the precedent dietary and the comparison phases. The objective was to test the hypothesis that when provided in equal amounts, the capacity of the rat to oxidize dietary linoleic (18:2n-6), α-linolenic (18:3n-3) and oleic acids (18:1n-9) is similar and independent of the degree of unsaturation.

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Abbreviations: FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids.

MATERIALS AND METHODS

Animals and diets. Thirty male Sprague Dawley rats (80–100 g; Harlan Sprague Dawley, Indianapolis, IN) were initially fed rodent Purina Laboratory Chow (Purina Mills, St. Louis, MO) and water *ad libitum*. Animals were housed at constant temperature (20°C) and humidity (54–56%) at a 12-h light–dark cycle throughout the experiment. After one week, rats were individually housed in steel cages and switched to an elemental diet containing 15% fat blended to contain equal proportions of linoleic, α-linolenic and oleic acids as triglycerides for ten weeks (Table 1). All oils were obtained from local sources. The equal proportion oil diet was prepared by blending sunflower, flax and olive oil, high in linoleic, α-linolenic and oleic acids, respectively, at a ratio of 1.11:3.00:0.92 (w/w/w). Within individual oils, weight ratios of linoleic, α-linoleic and oleic acids for sunflower, flax and olive oil were 0.456:0.007:0.153, 0.179:0.379:0.180, and 0.116:0.008:0.479, respectively. Overall fatty acid composition of the oil provided was 18:2n-6, 22.69%; 18:3n-3, 23.24%; 18:1n-9, 23.16%; 18:0, 2.48%; and 16:0, 5.88%. Butylated hydroxytoluene was added at a level of 0.02%, and diets were prepared every two to three days. Oils were stored at –5°C. The fatty acid ratio in the final diet was determined by gas–liquid chromatography (GLC). All procedures were approved by the Animal Care Experimentation Committee of the University of British Columbia (Vancouver, British Columbia, Canada).

Fatty acid oxidation. After ten weeks on the blended-oil diet, rats were randomized into three groups on the basis of weight. Food was removed eight hours prior to the experiment. Animals in each group were administered 20 μCi each of either [1-^{14}C]linoleic, α-[1-^{14}C]linolenic or [1-^{14}C]oleic acid (50–60 mCi/mmol; Amersham, Oakville, Ontario, Canada) mixed with 200 μL of the blended oil and given intragastrically *via* an oral catheter. Immediately thereafter animals were placed in a metabolic chamber, and $^{14}CO_2$ was collected for 10 min every hour for 12 h. Carbon dioxide from the metabolic chamber was collected by passing the air from the chamber through a sulfuric acid trap for drying, followed by two collection columns containing a mixture of methoxyethanolamine and ethanolamine (2:1, vol/vol) (6). Samples from the mixture were removed after each collection period and added to scintillation fluid. Radioactivity was assayed using a scintillation counter (Isocap; Nuclear Chicago, Des Plaines, IL).

The trapping efficiency of the methoxyethanolamine/ethanolamine mixture used to collect the CO_2 exhausted from the metabolic chamber was examined in a pilot study. It was determined that the saturation point of the mixture occurred at least 3 min after the 10-min collection period, thus ensuring $^{14}CO_2$ collection without losses.

Gas chromatography. The fatty acid composition of individual oils and of the blended oil diet was determined using a gas–liquid chromatograph (model 5890, Hewlett Packard, Palo Alto, CA) equipped with a flame-ionization detector and a 30 m × 0.2 μm i.d. SP-2330 (10%) capillary column using helium as carrier gas (1 mL/min). Fat was extracted from homogenized aliquots of the blended oil diet using chloroform/methanol (2:1, vol/vol), and the extracts were saponified with KOH and then transesterified with boron trifluoride/methanol (13). Hexane was added, and the mixture was shaken to partition the fatty acid methyl esters (FAME) into the hexane phase. The resulting FAME were injected into the GLC column at 160°C. After 8 min, the column temperature was increased at 2°C/min for 30 min then held constant for an additional 20 min. Chromatographic peaks were identified by comparison of retention data with those of authentic FAME standards (Supelco, Bellefonte, PA).

TABLE 1

Composition of Diet Containing Equal Levels of Linoleic, α-Linolenic and Oleic Acids

Nutrient	Content (g/100 g diet)
Casein	18.00
Sucrose	62.77
Fixed ratio oil[a]	15.00
DL-Methionine	0.23
Mineral mixture[b]	4.00
Vitamin mixture[c]	—[c]

[a]Oil contained equal proportions of linoleic, α-linolenic and oleic acids by combining sunflower, flax and olive oil in a ratio of 1.11:3:0.92, respectively.

[b]Contained calcium carbonate, 38.14%; copper sulfate · $5H_2O$, 0.048%; ferrous sulfate · $7H_2O$, 2.70%; magnesium sulfate · $7H_2O$, 5.73%, manganese sulfate · H_2O, 0.45%; potassium phosphate monobasic (KH_2PO_4), 38.90%; potassium iodide, 0.079%; sodium chloride, 13.93%; zinc chloride, 0.026%; and cobalt chloride · $6H_2O$, 0.002%.

[c]Contained (in g/kg of diet): vitamin A (50,000 IU/g), 0.040; vitamin D (85,000 IU/g), 0.0026; vitamin E (25,000 IU/g), 0.44; choline chloride, 1.65; menadione, 0.049; *p*-aminobenzoic acid, 0.111; nicotinic acid, 0.10; riboflavin, 0.022; pyridoxine hydrochloride, 0.022; thiamin hydrochloride, 0.022; calcium pantothenate, 0.066; biotin, 0.0004; folic acid, 0.002; inositol, 0.111; and vitamin B_{12}, 30 mg.

RESULTS

An initial study was carried out to test the palatability of the blended oil diet compared to diets containing the individual composite oils. Groups of rats (n = 5) consumed either the 15% (w/w) blended oil diet (Table 1) or chose between three food cups containing diets identical in macronutrient content with fats added either as sunflower, flax or olive oil. Figure 1 depicts the growth curves of groups fed both the blended oil and the three optional diets over a 22-d period. Body weight gains were similar across the groups, indicating good acceptability of the blended oil diet by the rat. Food consumption of animals on diets containing the blended oil was also similar to that of animals on diets containing the individual composite oils.

The body weights of the groups given ^{14}C-labelled linoleic (508 ± 22 g, mean ± SEM), α-linolenic (528 ± 23 g) and oleic (518 ± 26 g) acids were similar. Recovery of $^{14}CO_2$ over the 12-h interval post-dose is shown in Figure 2. ^{14}C appeared in expired air within in the first hour of collection with a rapid increase reaching a peak at two to three hours for all three ^{14}C-labelled fatty acids examined. Peak excretion rates for [1-^{14}C]linoleic (5.28 ±

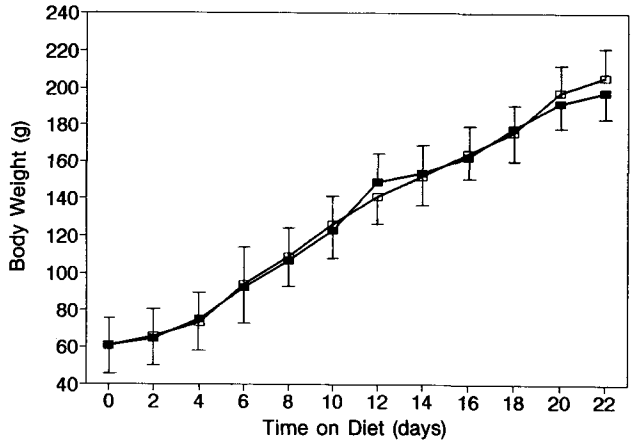

FIG. 1. Body weights of rats given either (*ad libitum*) a choice between three food cups containing identical diets with fat added either as sunflower, flax or olive oil (solid squares) or the blended oil 15% (w/w) fat diet (open squares) over 22 d; data are means ± SEM.

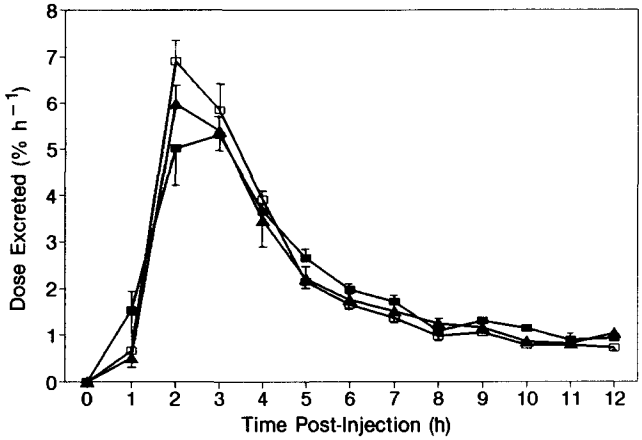

FIG. 3. Cumulative ^{14}C recovery as $^{14}CO_2$ over the 12-h interval post-dose after administration of 1-^{14}C-labelled linoleic (solid squares), α-linolenic (open squares) and oleic (solid triangles) acids; data are means ± SEM.

0.37%/h), α-[1-^{14}C]linolenic (6.92 ± 0.51%/h) and [1-^{14}C]-oleic (5.98 ± 0.44%/h) acids were not significantly different. There were no statistically significant differences in the oxidation rate at two or three hours. Each peak was followed by a gradual decline in excretion rate of label until 12 h; then a levelling off occurred at an excretion rate of about 1%/h.

Cumulative elimination rates for ^{14}C from rats given the labelled fatty acids are shown in Figure 3. There were no differences in cumulative excretion of label among [1-^{14}C]linoleic (27.2 ± 0.9%), α-[1-^{14}C]linolenic (26.8 ± 1.2%) and [1-^{14}C]oleic (25.9 ± 1.2%) acids over the 12-h duration, nor did any differences become significant when data were corrected for body weight.

DISCUSSION

The purpose of this study was to examine whether fatty acid oxidation rates would differ when both dietary and endogenous pool sizes were controlled. In previous studies, pool size often varied when fat oxidation was compared (6–10). The present work demonstrates that when the dietary pool size of the fatty acid examined is maintained at a consistent ratio prior to and during the oxidation period, ^{14}C elimination representative of oxidation rates is virtually identical for the fatty acids tested. These findings contrast results of a number of earlier studies with animals and humans that suggested that differences exist in whole body excretion of labelled fatty acids, but in these previous studies the labelled fatty acids were either provided in tracee pools of varying size, or the pretreatment diet was not standardized.

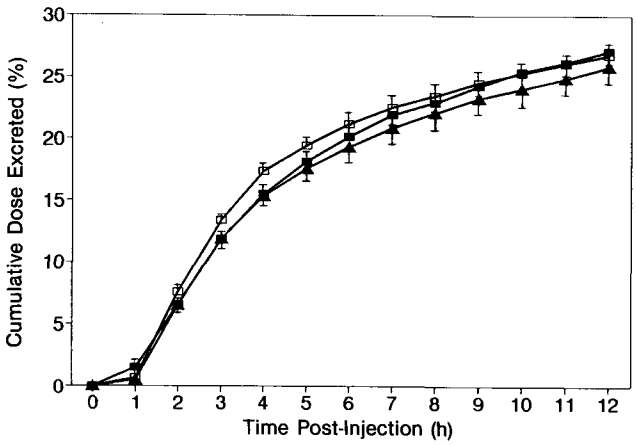

FIG. 2. ^{14}C recovery as $^{14}CO_2$ over the 12-h interval post-dose after administration of 1-^{14}C-labelled linoleic (solid squares), α-linolenic (open squares), or oleic (solid triangles) acid; data are means ± SEM.

In mice given ^{14}C-labelled oleic, linoleic and stearic acids transesterified with linoleic acid-rich corn oil (10), 50% of the administered label in oleic acid was recovered in expired CO_2 at 10-h post-administration, compared with 31 and 19% for linoleic acid and stearic acid, respectively. The comparative oxidation of oleic, palmitic and stearic acids was studied in rats (8) intubated with ^{14}C-labelled fatty acids transesterified with soybean triglyceride. Stearic acid ^{14}C appeared in expired air more slowly allowing 57% recovery after 51 h, compared to 66% from oleic acid. More rapid oxidation of linoleic as compared to stearic acid had been reported in rats fed each ^{14}C-labelled fatty acid mixed with olive oil (9). Similar studies in rats fed diets containing 15% of calories as triolein, trilinolein or tripalmitin, labelled with the respective ^{14}C-labelled fatty acid, had shown that 64, 52 and 51% of the dietary oleate, linoleate and palmitate, respectively, was oxidized after 72 h (14). Overall, these findings suggested differences in oxidation between MUFA, PUFA and SAFA; however, pool sizes of unlabelled (tracee) fatty acids likely varied widely between the fatty acids that were compared.

A similar oxidation pattern had been observed in humans (11). Subjects fed ^{13}C-labelled fatty acids mixed with a breakfast meal, exhibited greater 9-h cumulative $^{13}CO_2$ excretion in expired air from absorbed oleate (15.1%) than from absorbed linoleate (10.2%) or stearate (2.9%) (11). Data from fat malabsorption studies had shown that

orally ingested [^{13}C]triolein was converted to $^{13}CO_2$ more rapidly than [^{13}C]palmitic acid when given under the same conditions (15). Six hours after administration to healthy subjects, of each labelled fatty acid mixed in 0.7 g/kg of lipomul, 11.3 and 6.6% of label from triolein and palmitic acid, respectively, had appeared in breath. Other work has shown that triolein was oxidized more rapidly than tripalmitin when these ^{14}C-labelled fats were administered to healthy humans (16). However, when a comparison was made between whole body oxidation of orally fed ^{14}C-labelled oleic, linoleic and α-linolenic acids in healthy individuals after 24 h, no significant differences in $^{14}CO_2$ recoveries were observed (17). As with animal studies, the tracer/tracee ratio of specific fatty acids varied within the fat bolus fed. Thus, the findings of tracer studies carried out both in animals and in humans indicate that the shift of long-chain fatty acids toward oxidation, rather than storage, varies depending on the degree of unsaturation. However, without controlling fatty acid pool size, interpretation of the results of these studies remains difficult.

In other approaches aimed at examining whether fatty acid oxidation is related to structure, the tracer was directly introduced into the bloodstream, bypassing digestive and absorptive processes. When comparing whole body fatty acid oxidation, direct injection can compensate for potential variations in gastrointestinal absorption and dietary pool size between the various fatty acids tested. In rats maintained on formula diets and injected with ^{14}C-labelled linoleic, oleic and stearic acids, the oxidation rate as determined from cumulative $^{14}CO_2$ production was found to be highest for linoleic acid and lowest for stearic acid (18). The same group later reported increased oxidation of linoleic acid over palmitic acid (19), postulating that production of a highly metabolizable gluconeogenic propionyl-CoA from linoleic acid during oxidation explained the elevated rate of oxidation of linoleic acid. The oxidation rates of infused albumin bound [^{14}C]palmitic and [^{14}C]oleic acids were similar in dogs (20). Conversely, in humans, oxidation rates of injected ^{14}C-labelled palmitic, oleic and linoleic acids were 9.4, 10.0 and 6.3% of the dose administered, respectively, after one hour (7).

Although direct injection of tracer bypasses digestive processes, data interpretation in studies using injection when comparing dietary fatty acid oxidation can be difficult as the amount of injected label appearing in CO_2 represents the fraction of the plasma pool oxidized, not the absolute amount. Net or absolute oxidation measurement depends on the plasma pool size as well as the fractional turnover rate. Markedly smaller plasma pools of linoleic acid in comparison with palmitic acid have been reported (21). Thus, whether the appearance rate of a tracer directly corresponds to net fatty acid oxidation remains to be determined.

By contrast, other investigators have noted no difference in fatty acid oxidation rates. Awad *et al.* (22) found no difference in energy deposition between rats fed PUFA vs. MUFA/SAFA containing diets at 20% of energy for four weeks. Similarly, no difference was observed in humans in $^{14}CO_2$ excretion after oral administration of ^{14}C-labelled palmitic, stearic, oleic, linoleic and α-linolenic acids in healthy individuals after 24 h (17). Toorop *et al.* (12) controlled both the specific activity and net quantity of dietary linoleate and palmitate given to rats and found that, expressed relative to fatty acid absorbed, the oxidation rates of each of the fatty acids were similar. In other studies, such as those of Leyton *et al.* (6), enhanced conversion of α-[^{14}C]linolenic acid to $^{14}CO_2$, when compared to other fatty acids, would be expected given the very small pool size of linolenic acid within the olive oil carrier. In this case, although the proportion of α-[^{14}C]linolenic acid oxidized from the carrier oil was large, net oxidation would have been minimal.

During movement between gut and site of oxidation, labelled fatty acids must cross several transport and metabolite pools, the size of which will be at least partially dependent on prior dietary intake. Given two different labelled fatty acids entering this network of pools from the gut at equal rates, the fatty acid traversing a series of larger pools before arriving at the site where it is oxidized will become more diluted when compared to a fatty acid crossing smaller pools. This dilution will result in a diminished rate of labelled CO_2 expiration. Our findings suggest that a pretreatment diet containing equal fatty acid pools may correct for differences in oxidation due to variability across these internal fatty acid pools. When the labelled fatty acid is then delivered in an oil of equal fatty acid pools, each fatty acid behaves in a similar manner from the absorptive to the oxidative stages of metabolism. Previous work has suggested that absorption efficiencies of different MUFA and PUFA are similar in rats (23,24) and in humans (25), although transport from gut to liver may vary depending on chain length (26). Thus, it cannot be ruled out that absorption and oxidation kinetics varied between the fatty acids tested in a self-compensating manner. However, the similarity in the shape of the curves across the three fatty acids tested suggests similar kinetics.

In summary, the present data suggest that the capacity to oxidize fatty acids varying in degree of saturation is similar when rats are both prefed equal quantities of each fatty acid over a prolonged period and are administered the fatty acid at equivalent tracer/tracee ratios in a fat bolus. The results suggest that differences previously observed in fatty acid oxidation were due to the particular blend of fatty acids provided in the diet and not to the capacity of the organism to oxidize different fatty acids at different rates.

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Fatty Acid Turnover Rates in the Adipose Tissues of the Growing Chicken *(Gallus domesticus)*[1,2]

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The purpose of this study was to investigate the mobility of fatty acids in adipose tissue of the chicken and to determine whether adipose tissue dynamics are altered by dietary repartitioning agents. To this end, the turnover rates of fatty acids and triglycerides were estimated in adipose tissue of growing chicks by using isopentadecanoic acid (IPDA) and elaidic acid (EA) as marker dietary fatty acids. The half-life of IPDA in abdominal and sartorial adipose tissues of birds over 6 to 10 wk of age were 20 ± 4 and 23 ± 6 d, respectively. The half-life for the remaining total carcass lipids was 23 ± 3 d. The corresponding half-life for EA in abdominal fat tissue of birds over 2 to 7 wk of age was 18 ± 3 d, a half-life not significantly different from the IPDA half-lives. On the other hand, a thyromimetic repartitioning agent (L-94901) fed to birds at the 2 ppm level from 2 to 7 wk of age significantly decreased the half-life of EA in abdominal fat tissue to 6 ± 2 d. The data suggest that fatty acids were released from a more labile adipose site and subsequently reincorporated into abdominal and sartorial tissues and that fat mobilization occurred at the same time as did adipose tissue deposition in the growing chicken.
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Differences in fat deposition among breeds and strains of chickens point at the effects of genetic factors on body composition. Animals selected for fast growth and feed efficiency often show a propensity to deposit excessive amounts of fat (1–3), and genetic factors often affect body weight and tissue and plasma lipid composition. Also, the activities of lipogenic and lipolytic enzymes have been shown to significantly correlate with the genetic factors that favor high or low body weight (4–6). More recently, lipoprotein lipase activity and lipid content of abdominal fat tissue were used to predict the degree of fatness of broiler chickens (7).

Nutritional factors and the age of the birds also affect patterns of fat deposition and lipid metabolism (8–11). Other factors that control fatness in animals include feed restriction to slow fat deposition, and the use of growth promoters and partitioning agents to depress fattening and to enhance muscle accretion (12).

Triglyceride dynamics, such as fatty acid turnover, clearance, oxidation or uptake by adipose tissue have been investigated in humans and animals (13–15). Adipose tissue from the chicken serves as an excellent model for the study of direct incorporation and release of dietary fatty acids. The liver accounts for 90% of *de novo* fatty acid synthesis in young chickens (16), and other studies confirm that in birds hepatic lipogenesis is much greater than adipose tissue lipogenesis. Accordingly, nearly all fat that accumulates in broiler adipose tissue either evolves from synthesis in the liver or derives from the diet (17).

Among the methods used to measure lipid turnover, *in vivo* techniques using structurally isomeric fatty acids that occur naturally in trace amounts have often been used. To this end, we reported in a preliminary account of this work that isopentadecanoic acid (IPDA) is a suitable fatty acid marker for triglyceride turnover rates in the adipose tissues of growing chickens (18). While this work was in progress, Lemarchal *et al.* (19) reported the use of elaidic acid (EA) for determining triglyceride turnover rates in 11- to 21-week-old chickens that had been selected for either fatness or leanness. Aside from our earlier report (18), no measures of fat turnover have been published for young chickens when adipose growth rate is most rapid.

This study reports the half-life of the fatty acid markers, IPDA and EA in adipose depots of growing chickens between 6 and 10 wk and 2 to 7 wk of age, respectively. The effect of the thyromimetic repartitioning agent, L-94901 on the turnover rate of elaidic acid in abdominal tissue of the growing chick from 2 to 7 wk of age also was measured.

MATERIALS AND METHODS

Synthesis of labeled oils. The method for large-scale synthesis of triisopentadecanoin [(tri-13-methyltetradecanoyl)-glycerol; 13-MTDG)] by acid-catalyzed condensation of 13-methyltetradecanoic acid (IPDA) with glycerol has been published (20). Trielaidin (TE) was prepared similarly from EA obtained by the isomerization of oleic acid (21). 13-MTDG (400 g) was blended with a commercially available unhydrogenated soybean oil (1 kg). Fatty acyl chains were randomized by heating the oil mixture to 80°C for 2 h in the presence of 0.4% sodium methoxide under a nitrogen atmosphere (22,23). Similarly, EA-labeled oil was prepared starting with TE. The randomized oils had the fatty acyl compositions reported in Table 1.

Animals and diets. In Experiment 1, forty female broiler chicks (Shaver Poultry Breeding Farms Ltd., Ontario, CA) were tagged with wing bands and fed commer-

[1]Presented in part at the XVIII World Poultry Congress, Nagoya, Japan, September 1988.

[2]Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: ANOVA, analysis of variance; EA, elaidic acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; IPDA, isopentadecanoic acid, 13-methyltetradecanoic acid; L-94901, 3,5-dibromo-3'[(6-oxo-1,6-dihydropyridazin-3-yl)methyl]-DL-thyronine; 13-MTDG, tri-13-methyltetradecanoyl)-glycerol; $t_{1/2}$, half life/lives; T_3, triiodothyronine; TE, trielaidin.

TABLE 1

Fatty Acid Composition of Oil Supplements and Dietary Lipids[a]

Fatty acids	Soy oil and IPDA[b]	Soy oil and EA[b]	Unlabeled diet[c]	Labelled diet A[d]	Labelled diet B[e]
14:0	0.15	0.16	0.09	0.08	0.12
iso 15:0[f]	22.20	—	—	11.73	—
16:0	7.90	7.91	11.48	10.70	7.80
16:1n-9	0.35	0.33	0.13	0.12	0.17
18:0	3.64	3.64	2.32	2.09	2.35
t 18:1n-9	—	18.04	—	—	8.45
c 18:1n-9	20.40	22.30	22.94	21.55	26.54
18:1n-7	1.68	1.80	0.70	0.62	0.77
18:2n-6	38.60	38.62	54.19	49.94	46.76
18:3n-3	4.83	4.75	6.77	1.70	2.31
20:0	0.32	0.34	0.41	0.37	0.44

[a]Lipids from the unlabeled and labeled diets were isolated by Soxhlet extraction with diethyl ether. Fatty acid compositions are expressed in wt% as determined by gas chromatography (see Materials and Methods section).
[b]IPDA, isopentadecanoic acid, added as tri(13-methyltetradecanoyl)glycerol; EA, elaidic acid, added as trielaidoylglycerol.
[c]Lipid composition of diet with 2% soy oil added to feed.
[d]Lipid composition of diet with 2% soy oil–IPDA mixture added to feed.
[e]Lipid composition of diet with 2% soy oil–EA mixture added to feed.
[f]Isopentadecanoic acid, 13-methyltetradecanoic acid.

cial starter ration until the diet was changed to unlabeled diet formulation at eight days of age. The unlabeled diet (3.1 kcal/g; 23.1% crude protein; 11.9% moisture; 4.1% crude fat; 5.8% ash; 50.9% nitrogen-free extract; 2.3% crude fiber) was given *ad libitum*. At 25 d of age, birds were weighed and housed in individual cages equipped with individual feeder trays. At 28 d of age, birds were weighed and given individual feeders containing a fatty acyl-labeled diet (Diet A). The composition of the two diets was the same, except that labeled diet was formulated with 2% soy oil containing 22.2% of its triglyceride fatty acids as IPDA (soy oil and IPDA), which replaced 2% unlabeled soy oil in the unlabeled diet (Table 1). After two weeks of feeding the labeled diet, feeders were weighed and replaced with clean feeders containing unlabeled diet.

Experiment 2 was designed similarly to Experiment 1 with the exceptions that younger birds were used, and EA was the label. Two groups of 35 birds each were raised as described in Experiment 1 until birds were placed in individual cages at 14 d of age. A control group of 35 birds was given feeder trays containing a diet (Diet B) that was formulated as the unlabeled diet except that 2% soy oil was replaced with 2% soy oil that contained 18.0% of its triglyceride fatty acids as EA. Fatty acyl composition of the labeled oil (soy oil and TE) and the lipid isolated from the unlabeled and EA-labeled diet (Diet B) are given in Table 1. Feeders for the treated group contained the same labeled diet to which was added 2 ppm thyromimetic repartioning compound L-94901 (Smithkline Beecham Animal Health Products, West Chester, PA). Feeders of control and treated birds were removed after two weeks of feeding the labeled diet. Control birds subsequently were given feeders that contained unlabeled diet while treated birds were given feeders containing unlabeled diet with 2 ppm of L-94901.

Experimental design. In the IPDA study, Experiment 1, four birds were killed at 28, 33, 38, 42, 49, 56, 63 and 70 d of age. These ages correspond to 0, 5, 10 and 14 d of consumption of labeled diet and 7, 14, 21 and 28 d of subsequent consumption of unlabeled diet. Birds were killed by exsanguination after electrical stunning. Carcasses were chilled for 1 h in ice water immediately after scalding, defeathering and weighing. Abdominal and sartorial fat depots were excised and frozen at –20°C before lipid analysis. After removal of ingesta from the gastrointestinal tract, residual carcasses (defeathered carcasses without the removed adipose tissues) were ground with a meat grinder, and homogeneous samples were frozen for lipid analysis.

For both the treated and control birds of Experiment 2, feed consumption and body weight were recorded at least every other day from 14 d of age until death. Three birds from each group were killed at day 14, 18, 21, 28, 35, 39, 42 and 49 of age. These ages correspond to 0, 4, 7, and 14 d of consumption of labeled diet with (treated) or without (control) L-94901 and 7, 11, 14, and 21 d of unlabeled diet with (treated) or without (control) L-94901. Abdominal adipose tissues were collected and stored using the procedures described in Experiment 1.

Lipid isolation and analyses. Lipids were extracted from the diets with diethyl ether in a Soxhlet apparatus. Lipids were extracted from samples of abdominal (2 g) and sartorial (1 g) fat tissues, and residual carcass (5 g) by the method of Marmer and Maxwell (24). All tissue samples were analyzed in duplicate. Lipid extracts were stored in dichloromethane, and aliquots taken for percent lipid determination (wt/wt) and fatty acid composition.

Fatty acid compositions (Table 1) were determined by gas–liquid chromatography (GLC) of the fatty acid methyl esters (FAME). FAME were prepared and quantitated by the procedure of Slover and Lanza (25). FAME were separated on a Hewlett-Packard (Avondale, PA) Model 5890 gas chromatograph equipped with a split capillary injector, a flame-ionization detector and a fused silica capillary column, 60 m × 0.25 mm i.d., coated with SP-2340 (Supelco, Bellefonte, PA). He served as carrier gas (linear velocity of methane, 22.9 cm/s) at a split ratio of 80:1. FAME were separated by oven temperature programming: initial temperature, 140°C; then 0.5°C/min to 150°C; then 2°C/min to 200°C; hold for 20 min. Methyl heneicosanoate (21:0) served as internal standard. Signal analysis was accomplished by routing the detector output to a Hewlett-Packard Model 3396A integrator and a Hewlett-Packard Model 9122C mass storage unit for subsequent statistical analyses. The described chromatographic conditions gave complete baseline separation of all the fatty acids listed in Table 1.

Statistical analyses. The weight percent label found in the various tissues were analyzed by analysis of covariance using body weight as a covariate to determine the effect of diet and age. Unadjusted means for each combination of diet and age were then subjected to a nonlinear regression analysis using an exponential decay model. Half-lives ($t_{1/2}$, the time required for label content to

decline by one-half) of IPDA or EA for each tissue were calculated from the coefficients of the exponential decay model (19) of the form $Y = Ae^{-bt}$, where Y is the amount of IPDA or EA at time t in days, A is the amount of IPDA or EA at the apex value, and b is the rate of decline of IPDA or EA. Statistical comparison of data from experiments were made by analysis of variance (ANOVA). All analyses were performed using the SAS software system (26).

RESULTS AND DISCUSSION

IPDA and EA were used to determine fatty acid turnover rates in adipose tissues of growing chickens. In Experiment 1, four-week-old chicks were fed an IPDA-labeled diet for two weeks and subsequently fed an unlabeled diet for a four-week period. In Experiment 2, two-week-old birds were fed an EA-labeled diet for two weeks and subsequently fed an unlabeled diet for a three-week period. Chicks in the EA study were also fed the triiodothyronine analog L-94901 to determine if thyromimetic effects could be detected in fatty acyl turnover rates (27,28). All formulated diets had 4% total lipid content with fatty acid compositions as listed in Table 1. Unlabeled diets had no detectable amounts of IPDA or EA. Fatty acyl profiles were similar for each diet except for the impact of IPDA and EA substitution. Nearly 12% of the acyl residues in Diet A were IPDA, while approximately 8.5% of the fatty acyl residues in Diet B were EA.

The abdominal adipose depot was selected for study since it is the largest discrete depot and is an early developing adipose site (29). A second fat depot studied in the IPDA experiment was the sartorial fat pad. Like the abdominal depot, it is a discrete, though smaller adipose site that is well-defined at hatch but is a later developing fat depot (30). Lipids extracted from the defeathered carcass with the abdominal and sartorial depots excised were also analyzed. Lipids extracted from the residual carcass were analyzed to follow fatty acid turnover in lipids throughout the chicken, predominantly skin-associated, intermuscular and liver lipids.

Carcass weight, adipose depot weight and total lipid content of depots increased with growth of the birds during the IPDA (Table 2) and EA (Table 3) experiments. The general trend was for the residual carcass, and for the abdominal and sartorial fat tissues to increase in absolute mass during the IPDA study (Experiment 1). In each case, proportional lipid content remained constant as the absolute amount of lipid increased with age and body weight of the birds. Fatty acid marker analysis was designed to account for this dilution of the label with increasing tissue size by using body weight as a covariant in the data analyses.

The concentration of IPDA in fat depots (Table 2) increased from an undetectable level at the start of Experiment 1 (28 d of age) to a maximum of 19.3 mg/g of tissue for abdominal fat pad, 13.0 mg/g of tissue for sartorial fat pad, and 2.0 mg/g residual carcass after 14 d of feeding the IPDA diet (42 days of age). During this time, the total amount of IPDA in the tissues increased until 508, 43 and 2,351 mg was present in the abdominal, sartorial and residual carcass depots, respectively. However, the maximum amount of IPDA in the depots was reached at a tissue collection time subsequent to IPDA-diet withdrawal. The total amount of IPDA increased to 652 mg in abdominal fat, 57.6 mg in sartorial fat and 2,695 mg in residual carcass at 49 d of age or 7 d after labeled-diet withdrawal. The percentage total IPDA increase between 42 and 49 d of age was larger for abdominal (28%) and sartorial (35%) depots than for carcass (15%). These data suggest differences in mobilization and deposition rates among fat depots. However, for all tissues, total IPDA content consis-

TABLE 2

Abdominal Adipose Deport, Sartorial Adipose Depot and Clean Carcass Weight, Lipid Content, Proportional Isopentadecanoic Acid (IPDA) Content and Total IPDA in Fed Chickens[a]

	Age (days)							
	28	33	38	42	49	56	63	70
Abdominal fat pad								
Weight (g)	10.6 (0.4)[b]	17.6 (0.4)	15.3 (1.0)	25.8 (1.2)	45.9 (1.2)	40.1 (1.3)	52.5 (3.3)	45.7 (2.7)
Lipid content (g)	8.3 (0.4)	14.5 (0.3)	12.4 (1.0)	22.0 (1.1)	40.2 (1.2)	35.5 (1.1)	48.1 (3.4)	38.2 (2.2)
IPDA (mg/g tissue)	0.0 (0.0)	9.5 (0.3)	15.6 (0.4)	19.3 (0.3)	14.4 (0.3)	12.0 (0.4)	9.3 (0.4)	6.6 (0.2)
Total IPDA (mg)	0.0 (0.0)	168 (6.5)	242 (19.0)	508 (18.0)	652 (11.0)	476 (11.0)	499 (41.0)	309 (23.0)
Sartorial fat pad								
Weight (g)	1.5 (0.3)	2.1 (0.2)	2.6 (0.5)	3.4 (0.4)	5.0 (0.8)	4.4 (0.6)	4.6 (0.3)	7.7 (1.2)
Lipid content (g)	1.3 (0.3)	1.8 (0.2)	2.0 (0.4)	2.9 (0.3)	4.2 (0.7)	3.6 (0.5)	4.0 (0.3)	5.8 (1.1)
IPDA (mg/g tissue)	0.0 (0.0)	5.5 (0.7)	9.7 (0.9)	13.0 (0.7)	11.3 (1.1)	9.0 (1.5)	7.3 (0.8)	4.1 (0.4)
Total mg IPDA	0.0 (0.0)	11.5 (1.4)	25.2 (4.8)	42.8 (3.2)	57.6 (14)	37.4 (2.5)	33.3 (3.9)	31.7 (6.3)
Carcass								
Weight (g)	622 (7.2)	803 (14.0)	1017 (13.0)	1197 (14.0)	1622 (24.0)	1845 (42.0)	1831 (107)	2113 (70.0)
Lipid content (g)	65.8 (3.3)	89.8 (3.3)	103 (4.3)	141 (4.4)	215 (13.5)	236 (15.0)	225 (20.0)	297 (25.0)
IPDA (mg/g tissue)	0.0 (0.0)	0.9 (0.1)	1.5 (0.1)	2.0 (0.1)	1.7 (0.1)	1.4 (0.1)	1.0 (0.3)	1.0 (0.1)
Total IPDA (mg)	0.0 (0.0)	702 (53)	1563 (68)	2351 (90)	2695 (180)	2505 (158)	2121 (169)	1864 (119)

[a]The 28-day-old birds were fed an IPDA-labeled diet for two weeks followed by four weeks of feeding an unlabeled diet.
[b]Data presented are means and SEM of at least three birds.

TABLE 3

Abdominal Adipose Depot Weight, Lipid Content, Proportional Elaidic Acid (EA) Content and Total EA Content of Control and Thyromimetic-Treated Chickens[a]

		Age (days)							
		14	18	21	28	35	39	42	49
Carcass									
Weight (g)	Control[b]	267 (17)	378 (34)	503 (12)	722 (102)	1195 (87)	1377 (57)	1399 (107)	1763 (74)
	Treated[c]	242 (17)	388 (10)	496 (17)	771 (29)	1247 (92)	1301 (52)	1455 (120)	1942 (105)
Abdominal fat pad									
Weight (g)	Control	5.0 (1.4)	7.7 (1.9)	10.5 (1.0)	18.1 (1.4)[d]	35.1 (1.8)[d]	39.7 (1.8)[d]	41.0 (6.9)	49.9 (6.5)
	Treated	2.0 (0.1)	7.6 (0.8)	9.4 (1.0)	14.4 (1.1)[d]	27.9 (2.1)[d]	31.5 (2.1)[d]	34.5 (3.4)	52.8 (9.9)
Lipid Content (g)	Control	3.8 (1.4)	6.4 (1.6)	8.4 (0.8)	15.4 (1.8)	30.3 (2.5)	38.1 (2.2)	36.8 (7.0)	42.0 (6.1)
	Treated	1.8 (0.1)	6.5 (0.8)	7.7 (0.9)	12.2 (0.9)	24.8 (1.8)	27.6 (1.6)	30.5 (3.2)	47.6 (8.6)
EA (mg/g tissue)	Control	2.8 (0.4)	7.2 (0.8)	10.1 (0.2)	11.5 (0.3)	7.3 (0.6)	6.3 (0.2)	5.5 (0.3)	4.1 (0.50)
	Treated	3.2 (0.3)	8.3 (2.0)	9.3 (0.5)	10.6 (1.5)	13.0 (1.7)	9.6 (1.2)	3.1 (0.9)	1.1 (0.1)
Total EA (mg)	Control	13.2 (1.6)	55.5 (15)	106 (11)	207 (22)	270 (30)[e]	250 (18)	226 (40)[e]	200 (24)[e]
	Treated	6.6 (0.4)	63 (8.2)	87 (10)	151 (19)	357 (22)[e]	303 (51)	105 (26)[e]	47.4 (8.5)[e]

[a]Data presented are means and SEM of at least three birds.
[b]The 14-day-old birds were fed EA labeled diet for two weeks followed by three weeks on unlabeled diet.
[c]Fed as control birds except that EA diet and unlabelled diet contained 2 ppm thyromimetic L-94901.
[d]Paired (control-treated) values within a column with the same superscript are significantly different at $P < 0.10$ level using analyais of variance (ANOVA).
[e]Paired (control-treated) values within a column with the same superscript are significantly different at $P < 0.05$ level using ANOVA.

tently declined after the peak IPDA content was determined at 49 d until the end of the study.

The occurrence of maximal IPDA content at 49 d indicated that incorporation of IPDA from ingesta in the digestive tract after removal of labeled diet was significant. Labeled feed was removed from all birds in the experiment before they were killed at 42 d. Ingesta were removed from gastrointestinal tracts before 42-d birds were processed for analysis. Birds remaining on the study for observations beyond 42 d (49, 56, 63 and 70 d) were allowed to digest and adsorb ingested labeled diet. The increased IPDA content can be explained by this factor when combined with the variation associated with individual birds killed at these times. Approximately 12% of fatty acids in the 5% crude fat of the labeled diet was IPDA. The average daily feed intake for broilers at 42 d of age is over 100 g per day. Accordingly, digestive tract contents can include substantial amounts of feed stored in the crop. This residual absorption potential when considered with the size of standard errors relative to the amount of IPDA increase between 42 and 49 d supports this explanation. The delay in maximal IPDA content perhaps also includes loss of dietary fat in transport to the liver and lipoprotein particles during exsanguination (31).

Gross trends of abdominal adipose mass, lipid content and label incorporation data from Experiment 2 were similar to those of Experiment 1 (Table 3). Carcass weight, adipose tissue mass and total lipid content generally increased from the start, two weeks of age, to the end, seven weeks of age, of the study for both control and thyromimetic-treated (2 ppm L-94901) birds. There were no significant differences in body weight or feed intake between control and treated birds. However, significant ($P < 0.10$) decreases in the size of the fat pads were detected for thyromimetic-treated birds as compared to control birds at 28, 35 and 39 d of age, which correspond to 14, 21 and 25 d of exposure to thyromimetic (Table 3). The triiodothyronine analog L-94901 was observed to markedly decrease the abdominal adipose tissue mass in broilers (Gyurik, R.J., unpublished results) but the mechanism of this effect has not been published. Since thyromimetic compounds increase lipid mobilization and tissue turnover, they should also up-regulate fatty acid turnover rates. Moreover, increased thyroid hormone-like activity decreases relative abdominal adipose tissue mass in chickens (32).

EA incorporation into control birds exhibited characteristics similar to those observed for IPDA incorporation in Experiment 1. The concentration of EA in the abdominal adipose tissue of control chicks increased to an apex of 11.5 mg/g at 28 d of age (Table 3). This apex of EA concentration occurred at the time of EA diet withdrawal and is consistent with the data from all fat depots from IPDA Experiment 1. On the other hand, the apex at an EA concentration of 14.6 mg/g was not reached in thyromimetic-treated birds until 35 d of age. This anomalous observation suggests that fatty acid adipose tissue dynamics are altered by thyromimetic treatment.

In general, there were no significant differences in the total amount of EA deposited into the abdominal depot of treated birds relative to the amount deposited in control birds over 14 days of feeding the labeled diet. However, significant ($P < 0.05$) differences in total EA content were found after removal of the label that also suggested altered adipose tissue dynamics. The maximal total amount of EA incorporated into the abdominal adipose tissue was seen in 35 days of age for both control and treated birds (270 and 357, respectively). These maxima of total EA content are significantly different ($P < 0.05$) (Table 3) and followed cessation of feeding fatty acid-labeled diet, just as observed in the IPDA experiment.

The previously emphasized differences in responses between thyromimetic-treated birds and control birds from Experiment 2, and the repletion data from Experiment 1 can be explained by altered lipid and tissue dynamics. The increased EA concentration that was seen later in thyromimetic-treated birds than in control birds would result from a reduced dilution rate of EA with unlabeled fatty acids that occurs as a normal consequence of growth. An increased turnover of deposited triglyceride in treated birds would be consistent with this observation. However, the greater accumulation of total EA in thyromimetic-treated birds relative to control birds (357 vs. 270 mg) suggests that thyromimetic-stimulated fat mobilization was followed by a disproportionate redeposition of fat in abdominal adipose tissue from other more labile pools. This phenomenon also was observed in Experiment 1 where higher proportional increases of IPDA incorporation after label withdrawal (42 vs. 49 d of age) occurred in abdominal and sartorial depot lipid relative to the higher incorporation observed in carcass lipid (28, 35 and 15%, respectively). These site differences could result either from modifications of the dietary fatty acid profile and the effect of *de novo* fatty acid synthesis by the liver before subsequent delivery to fat depots (33) or from mobilization of fatty acids from labile depots and redeposition in less labile depots. The thyromimetic triiodothyronine analog L-94901 also may have suppressed *de novo* triglyceride synthesis (28).

The depletion rates of IPDA and EA from adipose depots and carcass lipids were measured as a decline in total fatty acid marker content. The $t_{1/2}$ of fatty acids, IPDA and EA, were determined from functions of total mg fatty acid label in the tissue over the age of the birds. These data are presented graphically in Figures 1 and 2 for Experiments 1 and 2, respectively. Two stages of IPDA content are depicted in Figure 1. An IPDA loading phase occurred from 28 to 49 d corresponding to 7 d beyond which the IPDA-labeled diet was fed. A depletion phase followed from day 49 to 70 d of age when birds were fed the IPDA-free, unlabeled diet. Similar repletion (14 to 35 d) and depletion (35 to 49 d) phases were observed for EA over the course of Experiment 2 (Fig. 2). Measures of fatty acid dynamics can be obtained from data collected during the depletion phase (13–15) since IPDA and EA are used as energy sources like normal constituent saturated and unsaturated fatty acids.

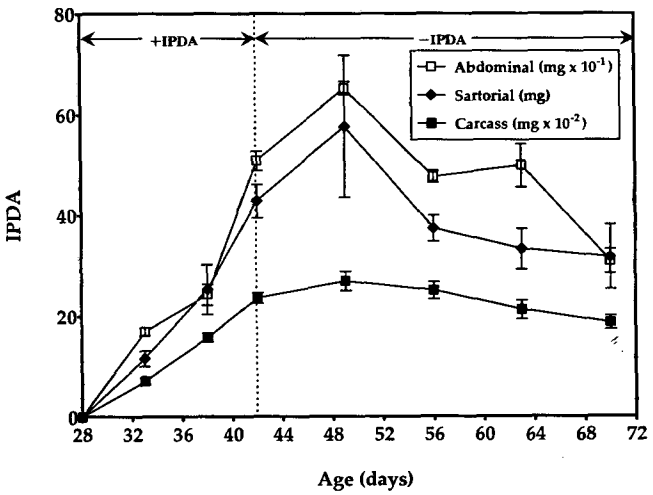

FIG. 1. Total isopentadecanoic acid (IPDA) content of adipose tissues (n = 3) as function of time for birds fed an IPDA-labeled diet from 4 to 6 wk of age followed by unlabeled diet to 10 wk of age.

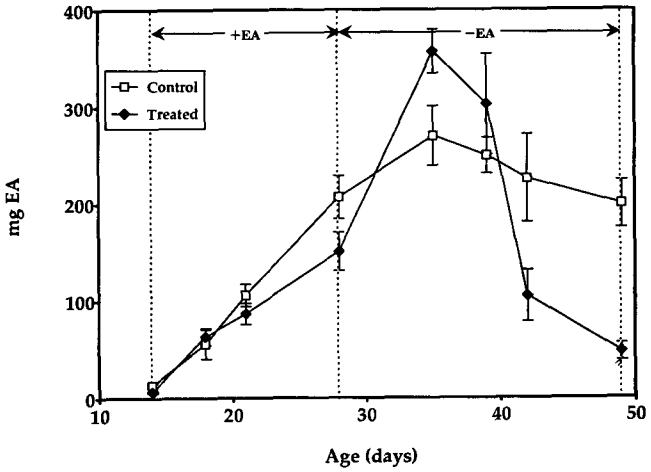

FIG. 2. Total elaidic acid (EA) content of abdominal fat pad (n = 3) as function of time. Control birds were fed an EA-labeled diet from 2 to 4 wk of age followed by 3 wk on unlabeled diet. Treated birds were fed the same diet as controls but containing 2 ppm of the thyromimetic L-94901.

The $t_{1/2}$ of IPDA and EA are listed in Table 4. For the abdominal and sartorial fat depots, $t_{1/2}$ of IPDA were 20 ± 4 and 23 ± 6 d, respectively. These values are not statistically different. For total carcass lipids, the $t_{1/2}$ of IPDA

TABLE 4

Half-Lives ($t_{1/2}$) and Turnover Times (T) for Isopentadecanoic Acid (IPDA) and Elaidic Acid (EA) in the Growing Chick

	IPDA		EA	
Tissue	$t_{1/2}$ (d)[a]	T (d)[b]	$t_{1/2}$ (d)[c]	T (d)[b]
Clean carcass[d]	23 ± 3	33 ± 5		
Sartorial[d]	23 ± 6	33 ± 9		
Abdominal[d]	20 ± 4	29 ± 6		
Abdominal (control)[e]			18 ± 3[f]	20 ± 2[g]
Abdominal (treated)[h]			6 ± 2[f]	9 ± 3[g]

[a]Half-life ± SE (days) of IPDA calculated from decay curve ($Y = Ae^{-bt}$, Y = total mg IPDA) from Figure 2.
[b]Turnover times of IPDA and EA were calculated from T = 1/*b* from decay curve $Y = Ae^{-bt}$.
[c]Half-life ± SE (days) of EA in days calculated from decay curve ($Y = Ae^{-bt}$, Y = total mg of EA) from Figure 3.
[d]Data for 28-day-old birds fed IPDA-labeled diet for two weeks followed by four weeks on unlabeled diet.
[e]Data for 14-day-old birds fed EA-labeled diet for two weeks followed by three weeks on unlabeled diet.
[f,g]Mean values within the same column with a letter in common are significantly different ($P < 0.05$) using analysis of variance contrasts.
[h]Birds fed as control except that EA-labeled diet and unlabeled diet contained 2 ppm of the tyromimetic L-94901.

was 27 ± 2 d, which is not significantly larger than the former half-lives. The $t_{1/2}$ for EA in abdominal adipose tissue of growing birds was 18 ± 3 and 6 ± 2 d for control and thyromimetic-treated birds, respectively. These $t_{1/2}$ are significantly ($P < 0.05$) different, which suggests that the thyromimetic L-94901 affects adipose tissue dynamics. The $t_{1/2}$ for IPDA determined in the adipose depots and carcass lipids of Experiment 1 are comparable to those reported for the rat in epididymal fat ($t_{1/2}$ = 18 d) (13) and for adipose tissue using other structurally isomeric fatty acids (34). In Experiment 2, the $t_{1/2}$ of elaidic acid found in abdominal tissue of control birds (18 d) was lower than the reported $t_{1/2}$ of EA in abdominal tissue for 11- to 21-week-old-chickens (29 d) reported by another laboratory (19). This difference may be due to the more rapid development and tissue turnover rates of fat depots in two- to seven-week-old birds as compared with older birds.

Turnover times (T), defined as the time required for a given amount of given fatty acid triglyceride to be removed from an adipose site, were calculated from the relationship $T = t_{1/2}/\ln 2 = 1/b$ where b is the slope of the exponential curve used to determine the $t_{1/2}$ of the fatty acid. This relationship assumes no exogenous source of the fatty acid during the measurement of the depletion phase of the study. Under this condition, T for IPDA and EA were measured as reported in Table 4. As with $t_{1/2}$, no differences in T for IPDA in the adipose tissues studied were observed with T on average being 30 d (Table 4). The T for EA in abdominal adipose tissue were statistically different ($P < 0.05$) for untreated and treated birds (20 ± 2 vs. 9 ± 3 d, respectively). These times are shorter than those found (18) for older birds where a T of 43 and 46 d was reported. The data indicate that coincident with rapid growth and fat depostion, significant amounts of triglyceride are mobilized in the growing chick. The data also suggest that the T3 analog L-94901 significantly affects the turnover rates of fatty acids in abdominal adipose tissue and affects fat pad growth.

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Effects of Dietary Fat and Fatty Acids on Sterol Balance in Hamsters

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Sterol balance studies, using both isotopic and chromatographic techniques, were carried out in hamsters fed semipurified diets to detect changes in sterol metabolism during the early period of the lithogenic stimulus. The balance studies examined animals in the first two weeks on the experimental lithogenic diets. The variables were as follows: dose of cholesterol (group 1, 0.05% vs. group 2, 0.2%); dietary fat (fatty acid) (group 2, butterfat vs. group 4, palmitic acid); source of hamster [group 2, Sasco (Omaha, NE) vs. group 3, Charles River (Wilmington, MA)]; average weight of animals (group 4, 60 g vs. group 5, 119 g). Animals in groups 1, 2, 3 and 5 maintained almost constant weight throughout the two-week balance study. Liver and plasma cholesterol levels increased in groups 2–5 with increasing dose of dietary cholesterol. The highest levels were found in group 4 (liver cholesterol, 32.7 mg/g; plasma cholesterol, 367 mg/dL). Sterol balance measurements showed that bile acid synthesis remained low (range 0.55–1.01 mg/d) for all groups regardless of the intake of dietary cholesterol (range, 3.27–20.90 mg/d). The dietary cholesterol absorbed from the intestine (range, 2.91–18.91 mg/d) was stored in the liver; this storage was reflected in the negative values for cholesterol balance for all groups (range, –0.70 to –14.97 mg/d). These studies did not reveal any correlations between parameters of sterol balance and cholelithiasis.

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The etiology of cholesterol cholelithiasis is probably multifactorial–metabolism of biliary lipids and proteins, the gallbladder and, indirectly, the diet all play a role in producing supersaturated bile, nucleating factors, vesicles, cholesterol crystals and stones (1–9). The systematic examination of factors believed to be important for the initiation/propagation of cholesterol cholelithiasis requires an effective and reliable animal model. Our laboratory has used the Sasco hamster (Omaha, NE), fed a nutritionally adequate semipurified diet (SPD) containing corn oil plus 4% butterfat and 0.3% cholesterol, for several reasons. First, animals fed this diet consistently had a cholesterol gallstone incidence of 50–60%, remained healthy and continued to grow (10–13). This was in contrast to animals fed fat-free lithogenic diets, which also form stones; however, many animals developed diarrhea, became ill and died during the experiment (14).

We have reported that cholesterol gallstones can be influenced by several factors, including (i) the intake of various dietary fats (12,13) and (ii) the source of hamster. A group of Sasco hamsters fed the SPD plus 0.3% cholesterol and 4% butterfat had a gallstone incidence of 63% (12). Replacement of butterfat with either olive oil, corn oil, menhaden oil or safflower oil prevented gallstone formation, whereas substitution of palmitic acid for butterfat intensified cholelithiasis (12). Animals from Charles River (Wilmington, MA), fed the identical lithogenic diet and treated in a similar manner, formed significantly fewer gallstones (10). The cholesterol metabolism/sterol balance in the hamsters from these two sources might be different, and this difference could alter cholelithiasis.

The sterol balance method was developed to provide a reliable estimate of cholesterol absorption, synthesis and turnover in the steady or near-steady state (15–23). The present study extends our preliminary sterol balance studies in hamsters (23) and examines in detail the effect of various factors as follows: (i) the effect of dose of cholesterol (0.05% vs. 0.2%) (group 1 vs. group 2), (ii) differences between strains (source) of hamster (group 2 vs. group 3), and (iii) cholesterol metabolism in animals with weights (ages) similar to those at the beginning (group 4) and at the end (group 5) of our gallstone prevention studies. Emphasis was placed on initial events (a two-week balance study) of our dietary manipulations prior to the development of cholesterol gallstones in this model. The data suggest that dietary fat (butter or palmitic acid) can produce an increase in plasma and liver cholesterol with little enhancement in endogenous bile acid synthesis. Cholesterol balance was a negative value, suggesting accumulation of dietary cholesterol in this model.

MATERIALS AND METHODS

Animals and diets. Male golden Syrian hamsters *(Mesocricetus auratus),* weighing either 57–66 g or 102–124 g, were purchased from Sasco, Inc. or from Charles River Breeding Lab. Animals were quarantined for one week after arrival. All animals (five per group) were placed in individual metabolic cages (Acme Research Products, Cincinnati, OH) for four days prior to the experiment, and fed powdered chow and water *ad libitum.* The cages used allow for quantitative recovery of feces from each hamster.

The methods were those commonly used for humans or rats, but with certain modifications necessary for the hamster (15–18). All food was finely ground and weighed every two days to allow accurate measure of food intake. On day 1 of the experiment, each hamster was injected intraperitoneally with 5 μCi of *R*-[2-^{14}C]mevalonolactone in sterile saline. Feeding of the experimental diet was begun immediately after the injection. All animals were maintained under similar conditions, using a 12-h light/dark cycle for the duration of the 14-d experiment. Feces were collected quantitatively on days 6, 8, 11 and 14 after isotopic labeling. Blood to measure the specific activity of serum cholesterol could not be collected *via* the tail vein as reported earlier (23), but was obtained by cannulation

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Abbreviations: GLC, gas–liquid chromatography; PAD, palmitic acid diet; SPD, semipurified diet.

(under local anesthesia) of the jugular vein (days 8 and 11) (followed by electrocautery to minimize bleeding) and by cardiac puncture (day 14). At sacrifice (day 14), samples of bile and liver were obtained for determination of biliary cholesterol specific activity, liver cholesterol concentration and liver cholesterol specific activity.

Labeled compound. *R*-[2-^{14}C]Mevalonolactone (Amersham Corp., Arlington Heights, IL) in toluene was found to be greater than 98% pure by thin-layer chromatography on silica gel 60 using acetone/benzene (1:1, vol/vol). Solvents were evaporated under nitrogen, and the mevalonolactone was redissolved in sterile saline (5×10^6 dpm/0.5 mL).

Reference compounds. Cholesterol (Sigma Chemical Co., St. Louis, MO) was recrystallized from boiling ethanol. The material was dried *in vacuo* and stored under nitrogen. 5α-Cholestane (Alltech Associates, Inc., Deerfield, IL) was used as the internal standard for gas–liquid chromatography (GLC) when separating the neutral sterols and acidic steroids after preparation of the trimethylsilyl derivatives.

GLC. The methods and conditions were similar to those described earlier, with certain modifications (15,16). All analyses were carried out on a Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph equipped with a flame-ionization detector. The column used was a capillary Econo Cap SE-30 (30 m, 0.32 mm) (Alltech). Conditions for the analyses, including injector (265°C), detector (265°C) and oven temperature (245°C) were identical to those used for packed columns (15,16).

Preparation of diets. The diets fed to all groups were purchased from Dyets Inc. (Bethlehem, PA). They contained the following basic components: 43.7% corn starch, 20% casein, 14.6% dyetrose (soluble starch), 10% fiber (cellulose), 5% salt mix (modified U.S.P. XIV salt mix no. 200951), 2% corn oil, 0.5% vitamin mix (no. 300000), 0.2% choline chloride and 4% butterfat. This diet is defined as semipurified diet + 4% butterfat, or butterfat diet. Cholesterol and 1.2% palmitic acid (in place of butterfat) was added and the groups/diets defined as follows: group 1, butterfat diet + 0.05% cholesterol; groups 2 and 3, butterfat diet + 0.2% cholesterol; groups 4 and 5, palmitic acid diet (PAD) + 0.3% cholesterol.

Methods for the isolation and quantitation of neutral and acidic steroids. The analyses were carried out using procedures described earlier (17). Fecal samples (0.8–1.0 g) were dried, powdered and extracted with ethanol for 48 h. After evaporation to 20 mL, 2 mL of 10 N NaOH was added, and the samples were refluxed for 1 h. Water was added, and the neutral sterols were extracted with hexane (50 mL, 2×). The neutral sterols were quantitated by GLC using 5α-cholestane as an internal standard after preparation of the trimethylsilyl derivatives. The weight of each neutral sterol component was directly proportional to the area of the 5α-cholestane (17).

The aqueous solution containing the acidic steroids was evaporated to dryness, redissolved in 18 mL of water and brought to 2 N in respect to NaOH by addition of 10 N NaOH. The acidic steroids were hydrolyzed at 14–16 psi in an autoclave for 3 h. Each sample was cooled and the pH adjusted to 1–2 by addition of HCl. The bile acids were extracted once with 50 mL of Folch mixture, $CHCl_3$/MeOH (2:1, vol/vol), followed by 50 mL of chloroform. All samples were evaporated to dryness. Bile acid methyl esters and trimethylsilyl derivatives were prepared using conditions as described earlier (17,18). Total bile acids were quantitated by capillary GLC using conditions similar to those for packed columns. The helium flow rate was 35 mL/min. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid was used as quantitative internal standard.

Calculations (Refs. 19,23). Fecal neutral sterols and fecal acidic steroids (mg/g, mg/d) were determined by GLC. Cholesterol in the diet was assayed following extraction of the food (1–2 g) with ethanol using procedures similar to those used for fecal neutral sterols. Endogenous neutral sterols (mg/d) were calculated from the value for the total radioactivity in the neutral sterol fraction divided by the specific activity of the plasma cholesterol determined two days earlier. Cholesterol absorption was determined by the difference between the intake of dietary cholesterol (determined from analyses of food) and the unabsorbed neutral sterols. Cholesterol turnover was the total of endogenous neutral sterols (mg/d) plus total (endogenous) bile acids (mg/d). Cholesterol balance was the difference between cholesterol turnover and cholesterol absorption.

Statistics. The numerical data are expressed as mean ± SD. Differences between data in experimental groups were calculated using analysis of variance to determine the F statistic. When the F statistic was significant, the level of significance was determined by Student's *t*-test (24).

RESULTS

Sterol balance studies were undertaken to determine whether diets that were used for gallstone formation (containing either butterfat or palmitic acid, a major fatty acid component of butterfat) altered cholesterol absorption/synthesis in the hamster. We fed nutritionally adequate semipurified diets that were altered to examine various parameters as follows: (i) cholesterol absorption by varying the amounts of cholesterol (0.05% and 0.2%), group 1 vs. group 2; (ii) cholesterol absorption by varying dietary fat (fatty acid) and dose of cholesterol, group 2 vs. group 4; (iii) comparison of a hamster in which stones form readily (Sasco) to one in which few stones form (Charles River), group 2 vs. group 3; and (iv) comparison of cholesterol balance in Sasco hamsters that are four weeks old (and weigh about 60 g) to those that are eight weeks old (and weigh about 120 g), group 4 vs. group 5. Groups 4 and 5 were studied because gallstone incidence was significantly higher in younger vs. older hamsters fed identical diets for six weeks. It would be important to determine whether factors affecting cholesterol metabolism were related to this difference. The balance period in these studies was for two weeks (in which stones and crystals had not yet formed) vs. six weeks for our gallstone formation studies. Our goal was to evaluate early changes in cholesterol metabolism using diets that affect stone incidence. Animals in all groups maintained almost constant weight throughout the experiment; daily food intake ranged from an average of 7.1 g/d (group 2) to 11.0 g/d

TABLE 1

Effect of Dietary Cholesterol and Fatty Acids on Animal Weight, Food Intake and Fecal Output[a]

Group	Source	Diet	Initial weight (g)	Final weight (g)	Food intake[b] (g/d)	Fecal output[c] (g/d)
1	Sasco[d] (four weeks old)	Semipurified diet + 4% butterfat + 0.05% cholesterol	106 ± 10 (99–116)	105 ± 11 (94–120)	7.8 ± 1.3 (5.5–9.8)	1.1 ± 0.2 (0.7–1.5)
2	Sasco (four weeks old)	Butterfat diet + 0.2% cholesterol	87 ± 8 (76–96)	95 ± 10 (86–104)	7.1 ± 1.9 (1.0–9.1)	1.0 ± 0.3 (0.4–1.3)
3	Charles River[d] (four weeks old)	Butterfat diet + 0.2% cholesterol	86 ± 4 (83–94)	84 ± 12 (66–96)	8.4 ± 2.3 (5.5–12.1)	1.2 ± 0.3 (0.6–2.1)
4	Sasco (four weeks old)	Palmitic acid diet + 0.3% cholesterol	60 ± 4 (57–66)	80 ± 20 (59–102)	8.5 ± 2.8 (2.7–13.0)	1.0 ± 0.3 (0.4–1.5)
5	Sasco (eight weeks old)	Palmitic acid diet + 0.3% cholesterol	119 ± 7 (112–124)	124 ± 12 (106–134)	11.0 ± 2.4 (6.7–14.0)	1.2 ± 0.4 (0.3–1.8)

[a]Numbers are averages ± SD; numbers in parentheses represent the range. Butterfat diet = semipurified diet + 4% butterfat; palmitic acid diet = semipurified diet + 1.2% palmitic acid. See Materials and Methods section for components of the semipurified diet.

[b]A daily average for the 14-day experimental period.

[c]Determined from the feces collected on days 6, 8, 11 and 14 of the experiment.

[d]Hamsters from Sasco (Omaha, NE) or Charles River (Wilmington, MA).

(group 5). Daily fecal outputs were almost identical in all five groups (range of 1.0–1.2 g/d) (Table 1). The specific activities of cholesterol in liver, plasma and bile were similar in each group on the day of sacrifice (day 14). Neither cholesterol crystals nor stones were present in the bile of any of the animals. Animals fed butterfat (groups 1–3) had similar weight gain, food intake, and fecal output and remained as healthy as animals fed the PAD (groups 4 and 5).

Tissue cholesterol levels are summarized in Table 2. Plasma cholesterol was similar in hamsters fed diets containing either 0.05% cholesterol (group 1) or 0.2% choles-

TABLE 2

Effect of Dietary Cholesterol and Fatty Acids on Cholesterol Concentration in Body Tissues and Gallstone Formation[a]

Group	Source	Diet	Liver weight (g)	Liver cholesterol (mg/g)	Plasma cholesterol (mg/dL)	Cholesterol gallstone incidence week 6[b]
1	Sasco (four weeks old)	Semipurified diet + 4% butterfat + 0.05% cholesterol	4.9 ± 0.7	6.1 ± 1.9[c]	192 ± 50	0/14[d]
2	Sasco (four weeks old)	Butterfat diet + 0.2% cholesterol	5.6 ± 0.7	17.4 ± 3.2	208 ± 26	8/16[e]
3	Charles River (four weeks old)	Butterfat diet + 0.2% cholesterol	6.1 ± 0.6	16.1 ± 4.8	212 ± 40	0/10[f]
4	Sasco (four weeks old)	Palmitic acid diet + 0.3% cholesterol	5.4 ± 1.4	32.7 ± 6.5	367 ± 97[g]	14/15[h]
5	Sasco (eight weeks old)	Palmitic acid diet + 0.3% cholesterol	5.8 ± 1.0	16.0 ± 2.3[i]	361 ± 82[j]	10/15[h]

[a]Numbers are averages ± SD. See Table 1 for information on diets and sources.

[b]Gallstone incidence determined from earlier studies after feeding experimental diets for six weeks.

[c]Differs from groups 2–5, $P < 0.01$.

[d]Gallstone incidence for animals treated similarly but fed semipurified diets + 4% butterfat + 0.1% cholesterol.

[e]From Reference 13.

[f]Gallstone incidence for animals treated similarly but fed butterfat diet + 0.3% cholesterol.

[g]Differs from group 1, $P < 0.01$.

[h]From Reference 26.

[i]Differs from group 4, $P < 0.01$.

[j]Differs from group 1, $P < 0.01$.

terol (group 2, 192 vs. 208 mg/dL, respectively). There was no significant difference in plasma cholesterol for Sasco vs. Charles River animals (group 2 vs. group 3). Plasma cholesterol levels increased significantly with the addition of 1.2% palmitic acid plus 0.3% cholesterol to the diet; the increase in plasma cholesterol (compared to animals fed 0.05% cholesterol) was similar when the starting weights were either 60 g (group 4) or 119 g (group 5; 367 vs. 361 mg/dL, respectively). Animals fed palmitic acid plus 0.3% cholesterol had plasma cholesterol levels higher than those fed butterfat + 0.2% cholesterol, regardless of age (367 and 361, groups 4 and 5 vs. 208 and 212, groups 2 and 3, respectively). Liver cholesterol levels increased significantly in groups 2–5 vs. group 1 ($P < 0.01$), with several interesting differences and similarities between groups. Young Sasco hamsters (group 4) had significantly higher cholesterol levels compared to older animals (group 5) given the identical diet. Liver weights were similar and ranged from 4.9 g (group 1) to 6.1 g (group 3). These weights tended to be higher in the cholesterol-fed groups (groups 2–5). No significant difference in liver cholesterol was observed in Sasco or Charles River hamsters fed the identical diet and treated under identical conditions. The incidence of cholesterol gallstones (after six weeks of feeding) was different depending on fat, source of hamster, as well as the amount of dietary cholesterol. We previously studied animals similar to those of groups 1 and 3 (10,13); these animals were given 0.1 and 0.3% cholesterol instead of 0.05 and 0.2% cholesterol.

The effect of the various diets on sterol balance is summarized in Table 3. Dietary cholesterol (g/d) was 3.27 (group 1), 11.70 and 11.90 (groups 2 and 3), and 18.70 and 20.90 (groups 4 and 5). These doses were chosen to enable us to detect differences in cholesterol absorption that could (after six weeks) affect stone incidence. Regardless of the amount of dietary cholesterol absorbed, daily acidic steroid output remained low for all animals in all groups (range 0.55–1.01 mg/d). Quantitation of acidic steroids was obtained from GLC measurements as no exogenous bile acids were included in the diet. Daily endogenous fecal neutral sterols were determined isotopically using the specific activity of plasma cholesterol two days prior to feces collection. In all cases, endogenous neutral sterols exceeded acidic sterol output 1.5- to 4-fold. This value was highest in older male Sasco hamsters (group 5, 3.13 mg/d) fed palmitic acid and lowest in Sasco males fed 0.05% cholesterol (group 1, 1.42 mg/d) or young Sasco males fed palmitic acid (group 4, 1.42 mg/d). Endogenous neutral sterols were similar in groups fed butterfat (groups 1–3) vs. palmitic acid (groups 4 and 5).

Cholesterol absorption ranged from 67% (groups 2 and 3) to 89–90% (groups 1 and 5). No differences in cholesterol absorption were noted based on source (groups 2 and 3 vs. group 1) or age of hamster (group 4 vs group 5). Animals fed the palmitic acid diets (group 5) absorbed more of the dietary cholesterol (18.91 mg/d, 91% of total dietary cholesterol) than other groups.

Cholesterol turnover (measured by combined isotopic and chromatographic methods) ranged from 1.84 mg/d to 3.94 mg/d. Cholesterol balance was a negative value in all groups, indicating a net absorption and storage of cholesterol in body pools.

DISCUSSION

The sterol balance technique was originally designed to estimate daily cholesterol synthesis in humans (15,16). The methods were modified so that they could be used in

TABLE 3

Effect of Dietary Cholesterol and Fatty Acids on Sterol Balance in Hamsters[a]

Group	Source	Diet	Daily acidic steroid output (mg/d)	Daily neutral sterol output (mg/d)	Daily endogenous sterol output (mg/d)	Daily cholesterol absorption (mg/d)	Daily cholesterol intake (mg/d)	Daily cholesterol turnover (mg/d)	Daily cholesterol balance (mg/d)
1	Sasco (four weeks old)	Semipurified diet + 4% butterfat + 0.05% cholesterol	0.86 ± 0.37 (0.35–1.66)	1.39 ± 0.36[b] (0.62–2.06)	1.42 ± 0.49 (0.77–2.28)	2.91 ± 0.39[c] (2.52–3.64)	3.27 ± 0.54[c] (2.29–4.12)	2.21 ± 0.60 (1.17–3.56)	−0.70 ± 0.40[c] [+0.52–(−6.25)]
2	Sasco (four weeks old)	Butterfat diet + 0.2% cholesterol	1.01 ± 0.45[d] (0.49–2.05)	4.51 ± 1.39 (1.84–6.31)	2.23 ± 0.98 (0.93–3.52)	7.93 ± 1.66[e] (6.45–10.93)	11.90 ± 3.22[e] (7.76–16.99)	2.90 ± 1.09 (1.40–4.26)	−5.02 ± 1.50[e] [−2.76–(−7.78)]
3	Charles River (four weeks old)	Butterfat diet + 0.2% cholesterol	0.64 ± 0.28 (0.25–1.28)	5.66 ± 1.65 (1.17–7.61)	2.41 ± 0.82 (1.20–3.72)	7.94 ± 0.98 (5.94–9.45)	11.70 ± 2.38 (7.52–16.36)	2.92 ± 0.90 (1.45–4.49)	−5.03 ± 1.14 [−3.65–(−7.27)]
4	Sasco (four weeks old)	Palmitic acid diet + 0.3% cholesterol	0.55 ± 0.31 (0.10–1.30)	3.65 ± 1.29 (1.75–6.31)	1.42 ± 0.67 (0.69–2.77)	13.37 ± 5.87 (3.51–17.57)	18.70 ± 6.06 (7.09–29.82)	1.84 ± 0.75 (0.92–3.45)	−11.53 ± 6.44 [−0.06–(−19.95)]
5	Sasco (eight weeks old)	Palmitic acid diet + 0.3% cholesterol	0.78 ± 0.39 (0.26–1.59)	8.64 ± 2.98[f] (2.59–13.92)	3.13 ± 1.85 (1.22–7.56)	18.91 ± 3.77 (14.72–23.67)	20.90 ± 6.66 (6.16–30.00)	3.94 ± 2.09 (1.53–8.78)	−14.97 ± 2.70 [−10.93–(−20.12)]

[a]Numbers are averages ± SD. See Table 1 for information on diets and sources. [b]Differs from groups 2–5, $P < 0.01$. [c]Differs from groups 2–5, $P < 0.01$. [d]Differs from group 3, $P < 0.02$. [e]Differs from groups 4 and 5, $P < 0.01$. [f]Differs from group 4, $P < 0.02$.

various animal models, such as the rat (17,18) and hamster (23). These animal models are useful for testing various synthetic and natural compounds using dietary modifications and administering new drugs in situations where human studies cannot be carried out. Our laboratory has reported that the hamster can form cholesterol gallstones in a reproducible and reliable manner in only 2–6 wk (10–13). The effect of the cholesterol diets (similar to those used to induce cholelithiasis) on total sterol balance has not been determined. The differences in stone formation observed between different hamsters might have their origin in differences in cholesterol metabolism. Consequently, we used the sterol balance method (modified for hamsters) to determine cholesterol absorption, endogenous sterol production and cholesterol balance in hamsters fed two lithogenic diets (butterfat + 0.2% cholesterol and palmitic acid + 0.3% cholesterol). The incidence of gallstones (after six weeks) when using butterfat + 0.2% cholesterol was almost identical to one using butterfat + 0.3% cholesterol (13). We felt it would be preferable to examine sterol balance at the lowest dose of dietary cholesterol where we could measure cholesterol absorption by using the methods of Quintão *et al.* (16).

Measurements of sterol balance are dependent on the following conditions (16)—constant plasma cholesterol concentration, unchanging fecal steroid output and constant body weight. All animals in our experiments fulfilled the first two criteria; constant body weight was maintained in all groups except in group 4 (the youngest of all groups at the start of the experiment). Nevertheless, group 4 is important for comparison because animals in this group are identical to those in which gallstone formation occurs within six weeks (10).

The measurement of endogenous neutral sterols required that we determine the specific activity of plasma cholesterol at three time points. In human studies, as well as in sterol balance studies in rats (where blood was obtained from the tail vein), this measurement is simple and required no special surgical procedures. However, we were unable to obtain blood from the tail vein of either Sasco or Charles River hamsters. Cardiac puncture frequently proved fatal. We abandoned these approaches and obtained blood via dissection of the jugular vein (under anesthesia) followed by electrocauterization to stop any excessive bleeding. No hamsters became ill or died as a result of this new procedure. The blood we obtained by this method was analyzed for cholesterol concentration and radioactivity in the usual manner. As in the rat, the specific activities of cholesterol in liver, plasma and bile were similar in all animals at sacrifice.

The balance studies were carried out for 14 d; this time frame enabled us to evaluate "early" changes in sterol metabolism and correlate these changes to stone development seen in previous studies (at six weeks) (10). Animals ingested an average of 8.6 g of food per day. Our earlier balance studies were carried out in rats (19) rather than in hamsters. There are several important differences between these two species—presence of a gallbladder in hamsters vs. none in rats, and ability of hamsters to absorb higher amounts of dietary cholesterol (24) and store this cholesterol in the liver as cholesteryl ester. In this regard, hamsters absorb more cholesterol per unit body weight than rats (19).

Animals in group 2 (Sasco males) and group 3 (Charles River males) were compared to determine whether source of hamster, which dramatically affected cholesterol cholelithiasis (10), also affected sterol balance. Acidic steroid output (mg/d) was higher in Sasco male hamsters compared to Charles River hamsters fed the identical diet (1.01 vs. 0.64 mg/d, $P < 0.02$). This value represents the daily bile acid synthesis in animals fed no exogenous bile acids and maintaining almost constant weight (25). When bile acid synthesis was calculated, values of 1.06 and 0.76 (mg/100 g animal/day), respectively, were obtained. The lower bile acid output in feces was associated with a lower biliary lipid concentration observed for the Charles River (Lakeview) hamster (10). The fecal bile acid output in the hamsters in this experiment fed SPD was considerably lower than that for the rat (17,18) and hamsters fed chow diets (23). The daily fecal output averaged 4–6 g/d for rats (17,18) and 2–3 g/d for hamsters fed chow diets. Fecal output in the current experiment averaged 1.0–1.2 g/d for hamsters fed SPD. We suggest that an increased absorption of bile acids in hamsters fed SPD resulted in an increased return of bile acids to the liver with decreased synthesis (as compared to chow diets) to maintain bile acid balance. The explanation, however, for the lower incidence of cholelithiasis in Charles River vs. Sasco hamsters was not immediately evident from differences in sterol balance.

Hamsters in all groups, with the possible exception of group 1, responded to the increased load of dietary cholesterol by absorbing this cholesterol and storing most of it in the liver. As a result, cholesterol balance (mg/d) was a negative value, reflecting no net synthesis but rather the storage of this dietary cholesterol. Presumably, cholesterol synthesis in groups 2–5 was completely inhibited by the doses of exogenous cholesterol fed to these animals. Interestingly, liver cholesterol levels were significantly lower for eight-week-old (group 5) vs. four-week-old (group 4) hamsters. This is reflected in the increased excretion of total neutral sterols and increased turnover of cholesterol in the older (group 5) animals. Because cholesterol absorption is higher for the older animals, it suggests increased mobilization of cholesterol prior to cholesterol excretion.

The balance studies attempted to elucidate any differences between animals fed palmitic acid + 0.3% cholesterol vs. those fed butterfat + 0.2% cholesterol. Daily acidic steroid output (synthesis in our studies) was significantly reduced with 1.2% palmitic acid in the diet (group 4) vs. animals where this component was not added (group 2) even though cholesterol absorption was almost doubled for group 2 vs. group 4. Interestingly, animals in group 4 excreted the least amounts of acidic steroids, neutral sterols and had the smallest cholesterol turnover (compared to animals in group 3 and in group 5). Animals in group 4, studied earlier under identical conditions and fed for six weeks, had the highest incidence of cholesterol stones (95%) (13). We have reported that animals fed palmitic acid + 0.3% cholesterol have elevated biliary cholesterol levels (when stones are present at week 6) com-

pared to animals fed butterfat + 0.2% cholesterol (4.99 vs. 3.70 mg/mL, respectively) (13). This suggests that the inability of the animals to compensate for the increased cholesterol load (by either fecal excretion or bile acid synthesis) may be a factor responsible for stone development.

In conclusion, the sterol balance method elucidated several important differences between the experimental groups, such as acidic steroid and neutral sterol output, and cholesterol turnover. Cholesterol absorption was greater than 50% (of cholesterol intake) in all animals. Net cholesterol balance was negative, showing an influx of cholesterol into body pools. There were no significant differences in sterol balance measurements in hamsters from Sasco as compared to those from Charles River. Whether observable differences exist with other fats or fatty acids remains to be elucidated.

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The Distribution of Brominated Long-Chain Fatty Acids in Sponge and Symbiont Cell Types from the Tropical Marine Sponge *Amphimedon terpenensis*

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The tropical marine sponge *Amphimedon terpenensis* (family Niphatidae, order Haplosclerida) has previously been shown to possess unusual lipids, including unusual fatty acids. The biosynthetic origin of these fatty acids is of interest as the sponge supports a significant population of eubacterial and cyanobacterial symbionts. The total fatty acid composition of the sponge was analyzed by gas chromatography/mass spectrometry of the methyl esters. Among the most abundant of the fatty acids in intact tissue were 16:0, 18:0 and 3,7,11,15-tetramethylhexadecanoic (phytanic) acid. In addition, three brominated fatty acids, (5*E*,9*Z*)-6-bromo-5,9-tetracosadienoic acid (24:2Br), (5*E*,9*Z*)-6-bromo-5,9-pentacosadienoic acid (25:2Br) and (5*E*,9*Z*)-6-bromo-5,9-hexacosadienoic acid (26:2Br) were also present. The three brominated fatty acids, together with phytanic acid, were isolated from both ectosomoal (superficial) and choanosomal (internal) regions of the sponge. Analysis of extracts prepared from sponge/symbiont cells, partitioned by density gradient centrifugation on Ficoll, indicated that phytanic acid and the three brominated fatty acids were associated with sponge cells only. Further, a fatty acid methyl ester sample from intact tissue of *A. terpenensis* was partitioned according to phospholipid class, and the brominated fatty acids were shown to be associated with the phosphatidylserine and phosphatidylethanolamine fractions that are commonly present in marine sponge lipids. The phosphatidylcholine and phosphatidylglycerol fractions were rich in the relatively shorter chain fatty acids (16:0 and 18:0). The association of brominated long-chain fatty acids (LCFA) with sponge cells has been confirmed. The findings allow comment on the use of fatty acid profiles in chemotaxonomy and permit further interpretation of LCFA biosynthetic pathways in sponges. The assignment of the sponge studied, which is currently placed as *A. terpenensis*, is being supported to some extent, but the species is unusual in having C_{25} fatty acids as the major constituent in this group. Other factors, such as season or microenvironmental conditions, may influence observed fatty acid composition which tends to reduce the usefulness of fatty acid profiles as markers in sponge chemotaxonomy.

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Marine sponges have attracted the attention of lipid chemists because they frequently contain unconventional sterols with nuclear or side chain modifications, or possess high levels of fatty acids characterized by very long chains (VLFA, C_{24}–C_{34}). A number of the fatty acids isolated possess unusual functional groups such as hydroxy, methoxy, acetoxy or halogen substituents. The physiological role of such unusual lipid constituents is unclear, but they certainly separate sponges from other eukaryotes (1–4). The tropical marine sponge *Amphimedon terpenensis* (family Niphatidae, order Haplosclerida), which has been the subject of extensive chemical and biochemical study by our group in recent years (5–9), possesses a lipid composition unique even by sponge standards. Three features distinguish this species: (i) the presence of bioactive diterpene isonitriles, notably the major metabolite diisocyanoadociane (**1**) (10); (ii) the presence of low levels of Δ5,7-sterols, with conventional side chains, relative to terpene levels (7,8); and (iii) the presence of brominated long-chain fatty acids (LCFA), e.g., **2–4**, which we identified by two-dimensional nuclear magnetic resonance (NMR) spectroscopy (9). The structures of lipids **1–4** are all summarized in Figure 1. The absence of unusual sterols, together with the novel terpenes, suggested that the terpenes may play a primary role in membrane function in this sponge. This hypothesis is supported by observations that the terpene **1** was found to be associated with sponge cell membranes isolated from *A. terpenensis* and purified by differential centrifugation (8). This association is, however, complex and the degree to which the terpene substitutes for sterols appears to be related to sponge cell type. Larger archaeocytes and spherulocytes, in particular, exhibited higher membrane terpene concentrations relative to the smaller pinacocytes and collencytes. A unicellular cyanobacterium, possibly *Aphanocapsa feldmanni*, and numerous strains of eubacteria were also present within the sponge, and in varying concentrations in separated cell lines, although no correlations of bacterial density with terpene concentration were evident.

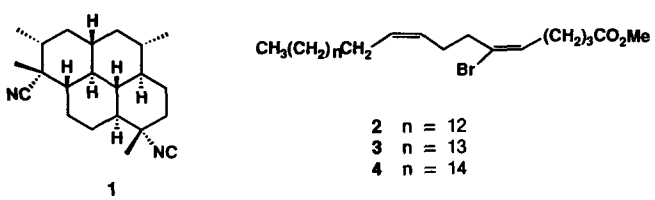

FIG. 1. Chemical structures of diisocyanoadociane (1) and selected fatty acids (2–4) from *Amphimedon terpenensis*.

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Abbreviations: CMF–ASW, calcium magnesium free artificial sea water; FAME, fatty acid methyl ester(s); GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LCFA, long-chain fatty acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TEM, transition electron microscopy; VLFA, very long-chain fatty acid.

The association of bacterial symbionts with marine sponges represents a major problem in utilizing sterol (11–13) and terpene (14) profiles in sponge chemotaxonomy. The continued discovery of novel terpene and sterol components is, however, of great physiological and pharmacological significance and generates intriguing hypotheses concerning their possible biosynthetic origin. The lipid content of *A. terpenensis* is interesting in a chemotaxonomic sense as it does not clearly distinguish the species affiliation. It was therefore deemed prudent to confirm the origin of the unusual brominated fatty acids **2–4** as being associated with sponge or symbiont cells. The membrane structure of *A. terpenensis* is perhaps unique and, therefore, this sponge is deserving of a more detailed fatty acid compositional analysis. The fatty acid content of the differing sponge and microbial cell types in *A. terpenensis* was therefore evaluated by the following three experimental methods: (i) separation of cyanobacteria by dissection of surface tissue; (ii) separation of phospholipid types according to class; and (iii) separation of dissociated cells, using methods recently developed for marine sponges (9,15–18) to yield preparations of low and high density sponge cell types. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Sponge collection. Seasonal variation in fatty acid content has been reported (4,19,20); therefore, all the samples used in this study were collected at the same time of year. Specimens of *A. terpenensis* (Australian Museum No. 4978) were collected near Townsville at -15 m from John Brewer Reef in November 1987 for intact tissue fatty acid analysis and preliminary cell separation. *A. terpenensis* was collected from Davies Reef in November 1989 for intact tissue fatty acid analysis, cell separation experiments and preparative isolation of fatty acids, and in November 1991 for analysis by phospholipid class. Sponges collected in November 1987 were place in aerated sea water maintained at ambient temperature while transported to the Sir George Fisher Centre for Tropical Marine Studies (Townsville, Australia), then placed in a small aquarium overnight at 18°C prior to the cell separation procedure. Samples selected for whole animal analysis were frozen for examination a few days later. Sponges collected in November 1989 were maintained in a flowing aquarium system at ambient temperature and with a normal light/dark regime at the Australian Institute of Marine Science for two days prior to cell separation. Unfortunately, this coincided with heavy sedimentation problems in this aquarium which effectively cut out all incident light. Sponges collected in November 1991 were maintained in a flowing aquarium system at ambient temperature and with a normal light/dark regime at the Australian Institute of Marine Science overnight before workup.

Chemicals and materials. Proteinase enzyme (No. P-2143), Ficoll type 400 and 14% BF_3-MeOH were purchased from Sigma Chemical Co. (St. Louis, MO). Calcium magnesium free artificial sea water (CMF–ASW) was prepared from doubly distilled water and contained 462 mM NaCl, 8.4 mM Na_2SO_4, 11 mM KCl and 2.1 mM $NaHCO_3$. All solvents were distilled prior to use or were of high-performance liquid chromatography (HPLC) grade (Mallinckrodt, Melbourne, Australia).

Cell separation. Method A: Dissection of whole sponge. Sponge tissue collected in November 1989 was carefully cleaned of all debris and separated into ectosomal tissue (66 g wet weight) and choanosomal tissue (70 g wet weight). A sample of intact animal tissue (380 g) was also retained for analysis. The three tissue samples were freeze-dried, then extracted and analyzed by gas chromatography (GC) for fatty acid methyl esters (FAME) as described previously (9).

Cell separation. Method B: Cell dissociation and density gradient separation. Cell separation of *A. terpenensis* was carried out on a sponge specimen (500 g wet weight, collected in November 1989) using previously described methods (8). The heavy sponge cell pellet (H_1) obtained by centrifugation of a dissociated sponge cell preparation at 600 × *g* at 10°C for 5 min (Damon IEC B-20A preparative centrifuge; Damon, Needham Heights, MA) was resuspended in a minimum volume of CMF–ASW and repelleted at 600 × *g* at 10°C for 5 min to remove bacterial and low density sponge cell contaminants prior to Ficoll treatment. The sponge cell pellet (H_2) obtained was divided into two portions; the major portion (90%) was resuspended in CMF–ASW, and cells were separated according to density by centrifugation (600 × *g* at 10°C) across discontinuous Ficoll gradients for 5 min. The bands of cells which accumulated at the interface between successive Ficoll fractions were isolated by pipette according to Figure 2, washed with CMF–ASW to remove Ficoll and pelleted (600 × *g* at 10°C for 5 min), then samples were taken for transmis-

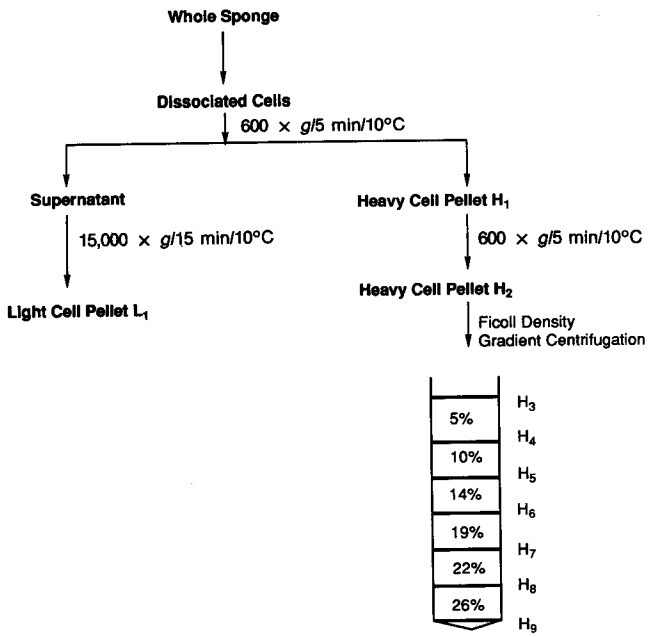

FIG. 2. Cell separation scheme using discontinuous Ficoll gradients. Capital letters with subscripts refer to cell pellets cited in text. Ficoll concentrations are weight percentage in calcium magnesium free artificial sea water.

sion electron microscopy (TEM). The two supernatants from H_1 and H_2 were combined and centrifuged at 15,000 × *g* at 10°C for 15 min to isolate a pelleted low density cell fraction L_1. The pellets H_3–H_9 and L_1 were lyophilized prior to chemical analysis. The minor portion of the sponge cell pellet H_2 (10%) was also lyophilized and then analyzed. Each pellet (approximate weight, 5–10 mg, except for H_7, <1 mg) was extracted with 3 × 1 mL of $CHCl_3$/MeOH (1:1, vol/vol). The soluble material was separated from other cellular debris by filtration through a small plug of glass wool and sand. The extract was dried under a stream of nitrogen, then treated with HCl-MeOH (1.4N; 1 mL) in screw-capped vials for 1 h at reflux. Solvents and reagents were azeotropically removed with toluene under a stream of nitrogen and the resulting material dissolved in toluene (0.25 mL) and passed through a 0.5 g florisil column eluting with toluene (3 mL). After evaporation of the solvent, the straw-colored extract was dissolved in hexane (0.25 mL) and passed through a 0.5 g silica (Anasil) column eluting with hexane (10 mL). The resulting clear solution was evaporated prior to GC analysis as before.

Analysis of sponge tissue by phospholipid class. A $CHCl_3$/MeOH (1:1 vol/vol) extract, prepared from 9 g of diced whole sponge (collected in November 1991), was concentrated *in vacuo*, dried azeotropically, and diluted to 15 mL with $CHCl_3$/MeOH. An aliquot (2 mL) was separated by preparative silica thin-layer chromatography under conditions ($CHCl_3$/MeOH/H_2O, 65:35:4, by vol) in which standard samples of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) gave R_f values of 0.76, 0.18, 0.55 and 0.46, respectively, using molybdenum blue for detection (18,21,22). Each phospholipid class was transmethylated with 1.4M HCl in MeOH and the resulting FAME mixture purified by Florisil chromatography, as described above, then analyzed by GC/mass spectrometry (GC/MS) using a Hewlett-Packard (Palo Alto, CA) 5890A instrument equipped with a 25 m (0.22 mm i.d.) bonded phase (BP_5) column (SGE, Melbourne, Australia) and a Hewlett-Packard 5970 series mass selective detector with helium as carrier gas.

Preparation of thin sections and electron microscopy. Whole animal tissue, ectodermal and choanodermal tissue, and cell pellets L_1 and H_2–H_9 were examined by TEM using methods detailed by Lawson *et al.* (22,23).

TABLE 1

Major Fatty Acids from the Phospholipids of *Amphimedon terpenensis*[a]

ECL	Fatty acid	Abundance	
14.00	Tetradecanoic (14:0)	0.3[b]	0.7[c]
14.62	13-Methyltetradecanoic (*iso*-15:0)	1.1	1.4
14.71	12-Methyltetradecanoic (*anteiso*-15:0)	0.3	[d]
15.00	Pentadecanoic (15:0)	0.3	0.8
15.63	14-Methylpentadecanoic (*iso*-16:0)	0.2	0.3
15.82	9-Hexadecenoic (16:1n-7)	1.4	3.5
16.00	Hexadecanoic (16:0)	5.9	13.9
16.42	Methylhexadecanoic (Me16:0)	1.9	2.1
16.63	15-Methylhexadecanoic (*iso*-17:0)	0.6	0.8
16.73	14-Methylhexadecanoic acid (*anteiso*-17:0)	0.4	1.1
17.00	Heptadecanoic (17:0)	1.5	1.0
17.61	5,9-Octadecadienoic (18:2)	0.3	0.6
17.70[e]	3,7,11,15-Tetramethylhexadecanoic	10.1	10.3
17.77	9-Octadecenoic (18:1n-9)	2.1	5.3
17.85	11-Octadecenoic (18:1n-7)	0.5	0.9
18.00	Octadecanoic (18:0)	2.4	2.7
18.41	Methyloctadecanoic (Me18:0)	3.1	2.9
18.63	17-Methyloctadecanoic (*iso*-19:0)	0.4	0.7
18.73	16-Methyloctadecanoic (*anteiso*-19:0)	0.3	[d]
19.00	Nonadecanoic (19:0)	0.9	0.8
19.40	5,8,11,14-Docosatetraenoic (20:4)	0.7	[d]
19.65	18-Methylnonadecanoic (*iso*-20:0)	1.6	1.7
19.73	17-Methylnonadecanoic (*anteiso*-20:0)	0.7	0.5
20.64[e]	19-Methyleicosanoic (*iso*-21:0)	1.0	1.0
20.74[e]	18-Methyleicosanoic (*anteiso*-21:0)	1.5	1.8
21.63[e]	20-Methylheneicosanoic (*iso*-22:0)	0.4	0.7
21.73[e]	19-Methylheneicosanoic (*anteiso*-22.0)	[d]	[d]
22.64[e]	21-Methyldocosanoic (*iso*-23:0)	[d]	0.6
22.74[e]	20-Methyldocosanoic (*anteiso*-23:0)	[d]	1.2
23.61[e]	5,9-Tetracosadienoic (24:2)	1.3	2.1
24.64[e]	5,9-Pentacosadienoic (25:2)	1.9	2.5
25.64[f]	5,9-Hexacosadienoic (26:2)	0.3	0.3
26.35[e]	6-Bromo-5,9-tetracosadienoic (24:2Br)	2.9	0.7
27.31[e]	6-Bromo-5,9-pentacosadienoic (25:2Br)	19.0	2.7
28.34[e]	6-Bromo-5,9-hexacosadienoic (26:2Br)	5.2	1.2

[a]By equivalent chain length (ECL). Minor peaks at ECL 17.06, 23.40, 24.30, 25.99, 26.80, 26.85 and 27.03 were not identified. Me16:0 and Me18:0 refer to mixtures of monomethyl-branched C_{17} and C_{19} fatty acids.
[b]November 1987 collection; HCl-MeOH.
[c]November 1989 collection; BF_3-MeOH.
[d]Less than 0.2% abundance.
[e]By ECL, nuclear magnetic resonance and mass spectrometry.
[f]By ECL and mass spectrometry.

RESULTS

Fatty acid analysis. The major FAME (C_9–C_{20}) were identified in standard fashion by establishing GC equivalent chain length values and by reference to the Hewlett-Packard Peak Library AEROBE, used previously for sponge fatty acid analysis (17,18,22,24). LCFA, >C_{20}, together with 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid), were also isolated by HPLC, and the methyl esters and *N*-acyl pyrrolides were analyzed by NMR and MS (9,25). A summary of the fatty acids detected in *A. terpenensis* is presented in Table 1. In November 1987, the most abundant fatty acids were found to be 16:0 (5.9%), 18:0 (2.4%), phytanic acid (10.1%), the brominated fatty acids (24:2Br, 2.9%; 25:2Br, 19.0%; 26:2Br, 5.2%) and branched chain acids such as Me16:0 (1.9%) and Me18:0 (3.1%). A FAME sample, prepared using BF_3-MeOH rather than HCl-MeOH, from sponge material collected in November 1989, gave much lower abundances for the brominated fatty acids.

Distribution of fatty acids—dissection of whole sponge. Tables 2–4 show the percentage abundance of fatty acids between whole animal, superficial and choanosomal tissue. The data shown in Table 2 indicate that the two major acids in *A. terpenensis* are 25:2Br and phytanic acid, respectively, and brominated acids 24:2Br and 26:2Br are also present in significant quantity. All three brominated acids **2–4,** together with phytanic acid, are

TABLE 2

Distribution of Major Fatty Acids in *Amphimedon terpenensis*[a,b]

Fatty acid methyl esters	Whole animal (%)		Surface tissue (%)		Internal tissue (%)	
	HCl-MeOH[a]	BF_3-MeOH[b]	HCl-MeOH[b]	BF_3-MeOH[b]	HCl-MeOH[b]	BF_3-MeOH[b]
16:0	5.9	13.9	9.4	17.5	11.6	9.7
18:0	2.4	2.7	1.7	2.7	2.6	1.8
Phytanic	10.1	10.3	11.7	9.2	21.7	11.2
24:2Br	2.9	0.7	1.7	1.2	3.7	0.6
25:2Br	19.0	2.6	10.0	5.7	26.9	5.6
26:2Br	5.2	1.2	3.6	2.3	8.2	1.4

[a]November 1987 collection. [b]November 1989 collection.

found in both superficial and choanosomal tissue. The brominated fatty acids are sensitive to the use of BF_3-MeOH, as indicated by their much lower abundances (contrasted with higher abundances for the saturated fatty acids) in extracts prepared with this reagent as compared to use of 1.4M HCl in methanol. The percentages listed should be taken as minimum estimates of brominated fatty acid in samples prepared with BF_3-MeOH.

Table 3 lists the percentage abundances in *A. terpenensis* of fatty acids normally attributable to cyanobacteria (26,27), in particular C_{16} and C_{18} saturated and unsaturated acids. *Amphimedon terpenensis* whole tissue contains high levels of C_{16} acids, both saturated and unsaturated, and the ratio of 16:0 to 18:0 is >1, as expected for a sponge rich in cyanobacterial symbionts (17). The major unsaturated acid present is 16:1n-7, in accordance with our identification of a unicellular cyanobacterial symbiont similar to *Aphanocapsa* sp. (7,8). If the data are normalized to a percentage abundance of 10% for phytanic acid [c.f. method of Gillan *et al.* (26)], the relative abundances of acids such as 16:0, 18:0 and 16:1n-7, which characterize the cyanobacterial symbiont of *A. terpenensis*, are seen to be more prevalent in the superficial tissue, where the cyanobacteria are localized, than in the choanosomal tissue.

Table 4 lists the distribution of fatty acids in *A. terpenensis* which are normally attributed to bacterial symbionts in marine sponges (26,28–30). Apart from the monomethyl-branched fatty acids Me16:0 and Me18:0, none of the fatty acids characteristic of bacteria are present in significant amounts in whole animal tissue of *A. terpenensis*; most are present in amounts less than those of the typical cyanobacterial acids described above. The low levels of individual bacterial acids are not unexpected, given the diversity of bacterial types detected within the sponge in our previous TEM study. The branched chain FAME, other than *iso*-15:0, *iso*-16:0 and *iso*-19:0, were evenly distributed between superficial and choanosomal tissue, consistent with our previous work which had demonstrated the presence of bacteria in both tissue types (9). Bacterial symbionts are therefore not present in large enough quantities to dominate the fatty acid composition of *A. terpenensis*. During the HPLC purification of brominated fatty acid esters, a number of branched LCFA, such as *iso*-21:0, *anteiso*-21:0 and their 22:0 and 23:0 homologues, were isolated in small quantity and characterized by NMR and MS.

Distribution of fatty acids according to phospholipid class. The major phospholipids in *A. terpenensis* were PE, PC, PS and PI. The PC fraction, characteristic of eucaryotes (18), contained the standard fatty acids 16:0, 18:0 and 18:1, but no LCFA. The PE fraction and, to a lesser extent, the PI fraction contained the triad of brominated acids, while phytanic acid was located in the PE and PI fractions, but not in the PS or PC fractions.

TABLE 3

Distribution of Fatty Acids in *Amphimedon terpenensis* Which Are Normally Attributed to Cyanobacterial Symbionts

Fatty acid methyl esters	Whole animal (%)		Surface tissue[a] (%)		Internal tissue[a] (%)	
14:0	0.3[b]	0.7[a]	0.3	0.3[c]	0.8	0.4[c]
16:0	5.9	13.9	9.4	8.0	11.6	5.4
16:1n-7	1.4	3.5	2.5	2.1	1.5	0.7
18:0	2.3	2.7	1.7	1.5	2.6	1.2
18:1n-9	2.1	5.3	3.8	3.3	1.5	0.7
18:2	0.3	0.6	0.7	0.6	[d]	[d]

[a]November 1989 collection. [b]November 1987 collection. [c]Normalized to phytanic acid at 10%. [d]Not detectable.

TABLE 4

Distribution of Fatty Acids in *Amphimedon terpenensis* Which Are Normally Attributed to Bacterial Symbionts

Fatty acid methyl esters	Whole animal (%)		Surface tissue[a] (%)	Internal tissue[a] (%)
*i*15:0[b]	1.0[c]	1.4[a]	[d]	0.6
*ai*15:0	0.3	[d]	[d]	0.8
*i*16:0	0.3	0.3	0.2	0.7
Me16:0	1.9	2.1	1.8	1.5
*i*17:0	0.6	0.8	0.4	0.5
*ai*17:0	0.4	1.1	0.9	0.4
18:1(11)	0.5	0.9	0.9	0.5
Me18:0	3.1	2.9	3.5	3.0
*i*19:0	0.4	0.7	0.3	[d]
*ai*19:0	0.3	[d]	[d]	[d]

[a]November 1989 collection. [b]*i* and *ai* refer to *iso* and *anteiso*, respectively. [c]November 1987 collection. [d]Not detectable.

No attempt was made to separate a phosphatidylglycerol fraction [characteristic of cyanobacteria and also the major phospholipid of gram positive bacteria (18)] from the PE fraction.

Distribution of fatty acids by cell type—cell dissociation and density gradient separation. Dissociation of sponge tissue and cell separation on the basis of density was accomplished using methods previously applied to *A. terpenensis* (8). As in the earlier work, the experimental conditions used damaged many cells, and small pellet samples were routinely obtained. Electron microscopy indicated that the dissociation process had yielded intact cells independent of the collagenous, extracellular matrix together with many cell fragments and other debris resulting from the dissociation process. Bacterial contamination of H_2, the dense sponge cell fraction, was low (less than 1%), whereas the low density cell fraction, L_1, was enriched in bacteria (6.4 ± 2.2%; mean ± SD) but also contained small sponge cells. After Ficoll separation of H_2, bacteria were located in H_3 (<1%) and H_5 (15.8 ± 1.3%). The specimen of *A. terpenensis* used contained lower levels of cyanobacteria than expected from previous work, possibly as a result of the sample having been stressed under the abnormal aquarium conditions. As a result, none of TEM samples H_1–H_9 showed significant levels of cyanobacteria; traces only were detected in H_2, H_6, and H_8. The fate of individual sponge cell types could not be determined completely as many of the pellets (H_3–H_9) contained much cell debris (10–30%, except for H_3 45%, H_5 66% and H_7 75%), most likely as a result of sponge cell lysis in the presence of proteinase in addition to disruption when in contact with Ficoll. The few intact cells in H_3–H_9 did not fix well, a problem that was also encountered in our previous work. Small rounded cells were noted in L_1, H_3 and H_4 (including unidentified vacuolated cells, possibly small spherulous cells) whereas H_6, H_8 and H_9 contained larger spherulous cells and archaeocytes. H_4 also contained archaeocytes. The only two fractions which were significantly different from those seen in our previous cell separation work were H_5 (containing many bacteria, no sponge cells, but possibly cell organelles and membrane material) and H_7 (with no sponge or symbiont types discernible).

Detailed chemical analysis was possible for all pellets created from Ficoll density gradient separation except H_7, for which the amount of pellet remaining after TEM sampling was very small. Extracts were methylated with MeOH-HCl and analyzed sequentially by GC using an automatic sampling program. Table 5 shows the percentage abundance of the major FAME in each cell preparation. The major fatty acid phytanic acid was present in all samples. There were larger amounts of phytanic acid in the denser sponge cell preparation (8.4% of FAME extract for H_2) as compared with the lighter cell preparation (5.1% of FAME extract for L_1); the highest

TABLE 5

Distribution of FAME in *Amphimedon terpenensis* According to Cell Type Isolated *via* Density Gradient Centrifugation[a,b]

	L_1	H_2	H_3	H_4	H_5	H_6	H_7[c]	H_8	H_9
					(%)				
Major FAME									
16.0	5.4	13.9	6.1	5.6	14.3	17.2	28.6	14.9	13.9
18:0	3.0	8.1	3.1	3.5	11.1	14.1	21.3	14.5	5.6
Phytanic	5.1	8.4	11.8	15.4	6.7	4.1	4.9	3.7	7.2
24:2Br	4.1	2.4	3.0	1.4	1.5	0.1	[d]	1.6	2.5
25:2Br	20.8	11.2	14.4	6.7	6.7	2.4	[d]	7.3	10.5
26:2Br	7.9	4.6	5.9	3.2	3.3	2.1	1.1	4.0	4.0
Other FAME attributable to cyanobacteria									
14:0	1.0	1.1	0.9	0.7	1.3	1.4	2.1	0.8	0.6
16:1n-7	0.8	[d]	1.0	3.7	3.8	4.0	3.7	2.5	1.8
18:1n-9	2.7	2.5	4.3	3.9	6.9	7.7	5.9	6.6	6.7
FAME attributable to (nonphototrophic) bacteria									
*i*15:0[e]	1.4	0.6	1.0	0.5	0.4	0.4	[d]	[d]	0.8
*ai*15:0	0.2	0.7	0.2	0.8	0.4	0.4	[d]	[d]	0.7
*i*16:0	0.3	0.3	0.4	0.2	0.3	0.3	[d]	[d]	0.4
Me16:0	1.7	1.2	1.5	1.5	0.9	0.7	[d]	[d]	1.8
*i*17:0	0.6	0.5	0.5	0.5	0.5	0.4	[d]	0.4	0.5
*ai*17:0	0.3	0.7	0.4	0.5	0.4	0.5	[d]	0.4	0.5
18:1n-9	0.8	0.3	1.3	1.1	1.2	1.1	[d]	1.1	1.1
Me18:0	2.2	2.3	2.0	2.3	1.2	0.9	2.1	0.8	2.0
*i*19:0	0.5	0.3	0.5	0.3	0.5	0.1	[d]	[d]	0.3
*ai*19:0	0.3	0.8	0.3	0.4	0.6	0.5	[d]	0.3	[d]

[a]November 1989 collection. FAME, fatty acid methyl esters. [b]See Figure 2 for identification of L_1 and H_2–H_9. [c]Small pellet size allowed detection of major FAME only. [d]Not detectable. [e]*i* and *ai* refer to *iso* and *anteiso*, respectively.

amounts of phytanic acid were located in H_3 and H_4 (11.8 and 15.4% of FAME extract as compared with 8.4% for H_2). The brominated fatty acids were also present in all cell preparations (percentages ranged from 20.8% of FAME extract for L_1 to 2.4% of FAME extract for H_6). The amounts of brominated acids in L_1 (for example, 20.8% for 25:2Br) were higher than the amounts in H_2 (11.2%), suggesting that the small rounded sponge cells in L_1 might contain these acids; when H_2 was partitioned by density gradient centrifugation, significant amounts of brominated fatty acid were present in fractions H_3–H_5. The pellets H_3 and H_4 had been identified by TEM as containing small rounded sponge cells. The electron micrographs also showed that H_3 contained insignificant numbers of eubacteria, H_4 was bacteria-free, while H_5 contained large quantities of bacteria. It is notable (Table 5) that cell pellet H_8, identified by TEM as a fraction containing archaeocytes, contained high levels of brominated fatty acids (7.3% for 25:2Br); therefore, heavier sponge cells may also contain these novel fatty acids. We attach little significance to the increased levels of brominated fatty acid in cell pellet H_9 as much of the material in this fraction represents reaggregated cells.

The amounts of 16:0, 18:0 and of other fatty acids, such as 16:1n-7, normally associated with cyanobacteria increased notably in H_5–H_8. Pellets H_6 and H_8 contained cyanobacteria as judged by TEM. The presence of cyanobacteria in H_5 and H_7 was not indicated by TEM, although the dark colors observed for these pellets (compared with cell preparations H_3 and H_4) was consistent with them containing cyanobacteria. The distribution of fatty acids usually attributed to bacteria in the sponge cell-enriched fractions H_3–H_9 was then compared with that in the bacteria-rich fraction L_1 (Table 5), and with the fatty acid composition of the whole animal (Table 4). Fraction L_1 contained amounts of typical bacterial fatty acids similar to those in whole animal, whereas these acids are generally present in smaller quantities in the dense cell preparation H_2, the only significant exception being *anteiso*-15:0, *anteiso*-17:0 and *anteiso*-19:0. The levels of Me16:0 and Me18:0 remained quite high in all the heavy cell pellets, but were not present in the same quantities as in whole animal.

DISCUSSION

There is now substantial evidence that marine sponges are sources of unusual fatty acids which are generally associated with the phospholipid fractions rather than other lipid fractions (1). In some recent cases, unusual fatty acids from marine sponges have been identified as membrane components (16,22–24). There have, however, been few studies on the lipid distribution according to cell type (16–18), even though an understanding of the cellular location of fatty acids is central to understanding their biosynthesis, their usefulness as chemotaxonomic markers and the physiological role of membrane constituents which potentially differentiate sponge cell membranes from other eukaryotic membranes. The major focus of our work was to determine whether the unusual triad of brominated LCFA were of sponge or symbiont origin. Cyanobacteria are not known to produce LCFA, and bacteria do not generally produce fatty acids with chain length >22:0 (28). Other possible symbionts, such as fungi or algae, were excluded from consideration as they were not apparent in light or TEM examination of sponge samples. The pigment patter for *A. terpenensis* is consistent with that elicited by a unicellular cyanobacterial symbiont and does not conform to any other algal type. Furthermore, brominated fatty acids have not yet been isolated from algae, although they have been isolated from a number of sponges of the orders Nepheliospongida, Haplosclerida, Halichondrida and Axinellida (25,31–41), each presumably with a different symbiont population. Sponges contain greater quantities of odd and branched fatty acids than do other plant or animal species, and contain the greatest range of unusual fatty acids of any phylum (1,19,20). All the brominated fatty acids isolated in our work possessed the Δ5,9*cis,cis* substitution pattern, which is characteristic of the VLFA ascribed to marine sponges (1,42). Therefore we hypothesized that brominated acids are located in sponge cells on both chemotaxonomic and phylogenetic grounds.

The experimental work described in this paper confirms that the suite of brominated fatty acids found in *A. terpenensis* are located in sponge cells rather than the diverse symbiont cell types associated with this sponge. Firstly, all three brominated acids were shown to be major components of both superficial and choanosomal tissues. The two tissue types both contain sponge cells, but differ in the nature of their symbiont populations. Fatty acids ascribed to cyanobacteria predominated in the superficial tissue whereas the range of fatty acids usually ascribed to eubacteria were present in low quantities in both superficial and choanosomal tissue, consistent with our previous electron microscopic identification of several morphologically distinct strains of eubacteria, each of which presumably has its own characteristic fatty acid composition. Secondly, the brominated fatty acids were found to be uniquely associated with the PE and PS classes. Lam *et al.* (25) isolated the C_{26} acid (**4**) from a Californian *Hymeniacidon* sp. and found that it was also a major component of the PE fraction. The association between sponge VLFA and amino-containing headgroups has been noted by other investigators (1,42,43). Finally, sponge cell preparations which were bacteria-free by TEM contained high levels of brominated acids. Fatty acids which are considered characteristic of bacterial or cyanobacterial symbionts showed distributions that were markedly different from those of the brominated acids in the cell preparations obtained; the fatty acid profile was generally consistent with the presence or absence of symbionts as determined by TEM.

The highest levels of brominated acids were associated with preparations (fractions L_1, H_3 and H_4) containing small sponge cells. Dissociated sponge cells commonly take on a more rounded shape than when present in whole tissue; this fact, together with the poor fixation which we have routinely encountered in our work on

A. terpenensis, prevented thorough morphological assignment of the cell types present. However, our earlier work has shown that these fractions might be expected to contain choanocytes and vacuolated cells (8), which together with the detection of increased levels of brominated acids in choanosomal tissue suggests that choanocytes may be the cell type which contains the brominated fatty acids. Although further analysis is required to confirm this possibility, our preliminary data are consistent with other reports in which purified choanocyte cells from *Tethya aurantia* and from *Pseudaxinyssa* sp. were shown to contain LCFA (17,18).

Biosynthetic experiments have shown that marine sponges can synthesize LCFA by chain elongation, followed by desaturation, of shorter chain (C_{14}, C_{16}) acids obtained from dietary sources or from symbionts (1). Precursor incorporation experiments by Lam *et al.* (25) on **4** established that bromination occurs at a late stage in the biosynthetic pathway, and this is consistent with the observation that brominated acids are of sponge origin as the chain elongation of short carbon chain fatty acid precursors is known to be carried out by the sponge host (1). The ability to introduce bromine into fatty acyl chains therefore appears to be a specific metabolic characteristic of sponge rather than of cyanobacterial or eubacterial cells. Bromination is most likely carried out on the preformed VLFA (perhaps by addition of Br_2 across a double or triple bond followed by elimination of HBr).

Phytanic acid is an isoprenoid fatty acid frequently isolated from marine sponges, but which has also been isolated from halophilic bacteria and is known to be transferred through marine food webs (43,44). In *A. terpenensis*, phytanic acid was shown to be a major constituent of the PE fraction. As aminophospholipids, such as PE and PS, have been found to predominate in sponge phospholipids (1,25), this suggested to us that phytanic acid is stored in sponge cells rather than in, or associated with, symbiont cells. Three other isoprenoid fatty acids have been isolated from marine sources. The fatty acid 4,8,12-trimethyltridecanoic acid has been demonstrated to be a component of the sponge cell population of *Pseudaxinyssa* sp., where it plays a structural role in membranes (17,22). This acid has also been useful as a taxonomic marker for sponges of the family Spirastrellidae (45). Carballeira and Maldonado (46) isolated a third isoprenoid acid, 3,7,11-trimethyldodecanoic acid, while Barnathan *et al.* (47) reported the isolation of 5,9,13-trimethyltetradecanoic acid. The increasing number of reports on the isolation of isoprenoid fatty acids from diverse sponge taxa with differing symbiont populations constitutes circumstantial evidence that these isoprenoid acids may be considered as a true component of sponge cells.

The use of fatty acids as a chemotaxonomic marker for sponges has been evaluated by Bergquist *et al.* (19), but is limited by seasonal, geographic and environmental variation (4,19,20). Furthermore, as our work, and that of others has shown, the fatty acid profile may be related to symbiont levels (18,26,42), although in many sponges the influence of symbionts may be minimal (19,48). Brominated fatty acids have been isolated previously from sponges of the families Nepheliospongidae (order Nepheliospongida), Hymeniacidonidae (order Halichondrida) and Agelasidae (order Axinellida) (25,31–41). The precise taxonomic status of the sponge used in this study is unclear as it shows certain taxonomic characteristics of the closely-related orders Nepheliospongida and Haplosclerida, but current information places the sponge as closest to the genus *Amphimedon* (order Haplosclerida) (49). Haplosclerid sponges are characterized by higher than average C_{27}–C_{30} acid levels, whereas sponges of the order Nepheliospongida are characterized by high levels of unsaturated LCFA, and by high C_{16} levels (19,20); some species are low in C_{17}–C_{23} acids (19). In its fatty acid composition, *A. terpenensis* shows many characteristics closely identified with nepheliospongids, but the sponge differs from other nepheliospongid species by having C_{25} as its major LCFA and in not containing measurable quantities of C_{27} acids. The levels of C_{25} acids in nepheliospongid species are usually lower than those of C_{27} acids (20). The recent report of brominated fatty acids from a Caribbean *Agelas* sp. (40) is of interest given that other sponges from this order contain isonitrile metabolites, as does *A. terpenensis*.

In an analysis of sponges of the genus *Amphimedon* from the Caribbean, Carballeira's group (50,51) found novel α-hydroxylated C_{22} and C_{23} acids, C_{23}, C_{25} and C_{27} monounsaturated acids together with C_{29} and C_{30} trienoic acids in *A. compressa*, while *A. complanata* was found to be rich in diunsaturated acids and in the rare eicosadienoic acids 6,11-20:2 and 11,15-20:2 (52). These two analyses differ significantly from those reported for the Australian *A. terpenensis*, highlighting the continued need to verify chemotaxonomic data with classical taxonomy and to examine further ecological influences on fatty acid variation within marine sponges.

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METHOD

Spectrophotometric Assay of 2,4-Dienoyl Coenzyme A Reductase with 5-Phenyl-2,4-pentadienoyl-Coenzyme A as Substrate

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The spectrophotometric assay of 2,4-dienoyl coenzyme A (CoA) reductase (EC 1.1.1.34) was modified to improve the linearity and sensitivity of this method. 5-Phenyl-2,4-pentadienoyl-CoA, which has an absorbance maximum at 340 nm with an extinction coefficient of 44,300 M^{-1} cm^{-1}, was synthesized and used as substrate. This compound is reduced by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 2,4-dienoyl-CoA reductase to 5-phenyl-3-pentenoyl-CoA. When a tissue homogenate serves as an enzyme source, the product is further metabolized by Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8) to 5-phenyl-2-pentenoyl-CoA, which is hydrated to 5-phenyl-3-hydroxypentanoyl-CoA by enoyl-CoA hydratase (EC 4.2.1.17). The modified assay method, which measures the decrease in absorbance at 340 nm due to the reduction of 5-phenyl-2,4-pentadienoyl-CoA and the oxidation of NADPH, is linear for a longer period of time and is twice as sensitive as the conventional assay with 2,4-decadienoyl-CoA as substrate.

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2,4-Dienoyl coenzyme A (CoA) reductase (EC 1.3.1.34) functions in the β-oxidation of unsaturated fatty acids by reductively removing even-numbered double bonds such as, for example, the 12-*cis* double bond of linoleic acid (1). Linoleoyl-CoA is chain-shortened by β-oxidation to 4-*cis*-decenoyl-CoA, which is converted by medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) to 2-*trans*-4-*cis*-decenoyl-CoA. The latter compound is reduced by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 2,4-dienoyl-CoA reductase to 3-*trans*-decenoyl-CoA which, after conversion to 2-*trans*-decenoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase (EC 5.3.3.8), can be completely degraded *via* the β-oxidation spiral. More recently, evidence was presented in support of a pathway by which odd-numbered double bonds, the 15-*cis* double bond of linolenic acid, for example, are also removed by 2,4-dienoyl-CoA reductase (2).

Further studies of 2,4-dienoyl-CoA reductase require a simple and sensitive assay method, especially as the specific activity of this enzyme in tissue extracts is low. The first report of a disorder of unsaturated fatty acid oxidation due to a deficiency of 2,4-dienoyl-CoA reductase (3) prompted the design of a sensitive radioactive assay of 2,4-dienoyl-CoA reductase (4). However, the need to synthesize [4*S*-^{3}H]NADPH for this assay limits its use. We therefore decided to improve the available spectrophotometric assay method, which is based on the measurement of NADPH oxidation at 340 nm, with either 2,4-decadienoyl-CoA or sorboyl-CoA as a substrate (5). By using 5-phenyl-2,4-pentadienoyl-CoA as a substrate, which has an absorbance maximum at 340 nm, the sensitivity and also the linearity of the assay method was improved.

EXPERIMENTAL PROCEDURES

Materials. Acyl-CoA oxidase from *Athrobacter* species, NADPH, CoA and other standard biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Aldrich (Milwaukee, WI) was the source of 2-*trans*-4-*trans*-decadienal, *trans*-cinnamaldehyde and 4-*cis*-decenal. The following enzymes were purified or partially purified by the indicated procedures: enoyl-CoA hydratase or crotonase (4.2.1.17) from bovine liver (6), 2,4-dienoyl-CoA reductase from rat liver (7) and peroxisomal trifunctional enzyme from rat liver (8,9). 4-*cis*-Decenal and 2-*trans*-4-*trans*-decadienal were oxidized with Ag_2O to 4-*cis*-decenoic acid and 2-*trans*-4-*trans*-decadienoic acid, respectively, by a general procedure for the oxidation of aldehydes to acids that are sensitive to strong oxidizing agents (10). 5-Phenyl-2,4-pentadienoic acid was synthesized by reacting *trans*-cinnamaldehyde with malonic acid in the presence of pyridine using a general procedure described by Linstead *et al.* (11). In brief, 2.5 g of *trans*-cinnamaldehyde were mixed with 2 g of dry pyridine and then combined with 2 g of dry malonic acid. The mixture was kept for 48 h at 25°C and then heated at 80°C for approximately 8 h until CO_2 ceased to evolve. The reaction mixture was acidified with 50% (vol/vol) H_2SO_4 and extracted with diethyl ether. The ether phase was extracted several times with 10% (wt/vol) Na_2CO_3. The aqueous solution was acidified with 50% (vol/vol) H_2SO_4 and extracted with diethyl ether. After drying the ether phase over anhydrous Na_2SO_4, the ether was removed under reduced pressure. 5-Phenyl-2,4-pentadienoic acid was obtained in approximately 50% yield. The acid crystallized at –20°C from petroleum ether (b.p. 37–52°C)/diethyl ether in the form of white needles with an m.p. of 166–168°C (literature m.p. 166–167°C) (12). CoA thioesters of 2-*trans*-4-*trans*-decadienoic acid, 4-*cis*-decenoic acid and 5-phenyl-2,4-pentadienoic acid were prepared by the mixed anhydride procedure as detailed by Fong and Schulz (13). Acyl-CoA compounds used for spectral or high-performance liquid chromatography (HPLC) analyses were purified by HPLC, whereas their use as substrates in enzyme assays did not require any purification. The concentrations of all acyl-CoA solutions,

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Abbreviations: A, absorbance; CoA, coenzyme A; HPLC, high-performance liquid chromatography; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; P_i, phosphate; U, unit; UV, ultraviolet.

including 5-phenyl-2,4-pentadienoyl-CoA, were determined by quantifying free CoA by the Ellman procedure (14) after completely cleaving the thioester bond with 1M hydroxylamine at pH 7 (13). 2-*trans*-4-*cis*-Decadienoyl-CoA was prepared from 4-*cis*-decenoyl-CoA by acyl-CoA oxidase under aerobic conditions as described previously (15), except that catalase was omitted from the incubation mixture. The ultraviolet (UV) spectrum of HPLC-purified 5-phenyl-2,4-pentadienoyl-CoA showed two broad peaks centered around 260 and 340 nm. The ratio of A_{340}/A_{260} was 1.95. The extinction coefficient of the absorbance peak at 340 nm was 44,300 M^{-1} cm^{-1}. Liver mitochondria were isolated according to a general procedure (16). For the preparation of a soluble extract, rat liver mitochondria were sonicated ten times for 8 s each with a Heat Systems (Plainview, NY) sonifier (Model W-385) equipped with a microtip and centrifuged at 105,000 × *g* for 1 h. The supernatant served as a source of soluble mitochondrial enzymes.

Assays of 2,4-dienoyl-CoA reductase and protein. 2,4-Dienoyl-CoA reductase was assayed spectrophotometrically by measuring the absorbance change at 340 nm with a Gilford (Oberlin, OH) Model 250 recording spectrophotometer at 25°C. Absorbance scans of the UV region were recorded with a microprocessor-controlled Gilford 2600 UV-VIS spectrophotometer equipped with a graphics plotter. The assay mixture (1 mL) contained 0.2 M KP_i (P_i, phosphate) (pH 8), 0.1 mM NADPH, 25 μM 2-*trans*-4-*trans*-decadienoyl-CoA or 25 μM 2-*trans*-4-*cis*-decadienoyl-CoA or 25 μM 5-phenyl-2,4-pentadienoyl-CoA and partially purified 2,4-dienoyl-CoA reductase (4–10 μg of protein), or a soluble extract of rat liver mitochondria (40–100 μg of protein) to give an absorbance change of 0.01 to 0.025 A/min. The assay was started by the addition of enzyme. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per min. Protein concentrations were determined by the dye binding assay as described by Bradford (17), with bovine serum albumin as standard.

HPLC analysis and purification of acyl-CoA. Prior to analysis by HPLC, incubations were terminated by acidification to pH 1–2 with conc. HCl. Samples were filtered through 0.22 μm (pore size) membranes, after which the pH was adjusted to 5 with 4N KOH. The filtrates were applied to a Waters (Milford, MA) μBondapak C_{18} reverse-phase column (30 cm × 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile content of the 25 mM ammonium P_i elution buffer (pH 5.5) from 20 to 80% in 20 min at a flow rate of 2 mL/min. When acyl-CoA was purified by HPLC, fractions containing the compound of interest were concentrated at room temperature on a rotary evaporator under reduced pressure generated by a water aspirator.

RESULTS AND DISCUSSION

2,4-Dienoyl-CoA reductase, which is an essential enzyme for the β-oxidation of unsaturated fatty acids, is routinely assayed by measuring the substrate-dependent oxidation of NADPH spectrophotometrically at 340 nm. Because the specific activity of this enzyme in tissue extracts is as low as 13 mU/mg in human liver (3), 4.6 mU/mg in human muscle (3) and 3.4 mU/mg in bovine liver (4), the sensitivity of the spectrophotometric assay with 2,4-decadienoyl-CoA as a substrate is frequently insufficient, especially when only small tissue samples are available. In an attempt to increase the sensitivity of the convenient spectrophotometric assay method, we set out to design and synthesize a substrate with an absorbance maximum close to that of NADPH at 340 nm and with a large extinction coefficient. 2,4,6-Octatrienoyl-CoA, which has an absorbance maximum at 337 nm with an extinction coefficient close to 50,000 M^{-1} cm^{-1}, was previously shown to be a substrate of 2,4-dienoyl-CoA reductase (7). However, this compound is not very stable and, hence, it was decided to synthesize and evaluate (as a reductase substrate) a compound that has a phenyl group in conjugation with the basic 2,4-dienoyl-CoA chromophore. These considerations prompted the synthesis of 5-phenyl-2,4-pentadienoyl-CoA, which has an absorbance maximum at 340 nm with an extinction coefficient of 44,300 M^{-1} cm^{-1} and which was found to be more stable than the commonly used substrate 2,4-decadienoyl-CoA. 5-Phenyl-2,4-pentadienoic acid was conveniently synthesized from *trans*-cinnamaldehyde and malonic acid in the presence of pyridine (11). Because the condensation reaction results in the formation of a *trans* double bond, 5-phenyl-2,4-pentadienoic acid prepared by this method was expected to have a *trans,trans* configuration. This assumption was confirmed by its melting point of 166–167°C, which is close to that reported for the *trans,trans* isomer (12).

When 5-phenyl-2,4-pentadienoyl-CoA was incubated with NADPH in the presence of 2,4-dienoyl-CoA reductase, the absorbance at 340 nm due to both substrates with a combined extinction coefficient of 50,500 M^{-1} cm^{-1}

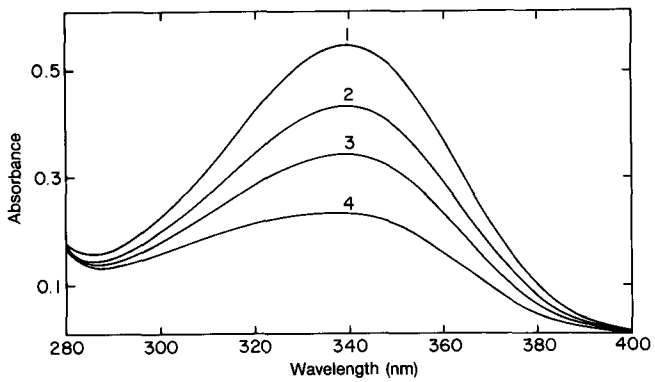

FIG. 1. Spectral changes associated with the reduction of 5-phenyl-2,4-pentadienoyl-coenzyme A (CoA) by nicotinamide adenine dinucleotide phosphate (NADPH) catalyzed by 2,4-dienoyl-CoA reductase. Curve 1, prior to the addition of 2,4-dienoyl-CoA reductase; curve 2, 3 min; curve 3, 8 min; curve 4, 30 min after initiation of the reaction. The reaction mixture contained 0.2 M KP_i (pH 8.0), 0.1 mM NADPH, 12.5 μM 5-phenyl-2,4-pentadienoyl-CoA, and partially purified 2,4-dienoyl-CoA reductase (15 μg/mL). The reference cuvette contained the same mixture, except that 5-phenyl-2,4-pentadienoyl-CoA was omitted.

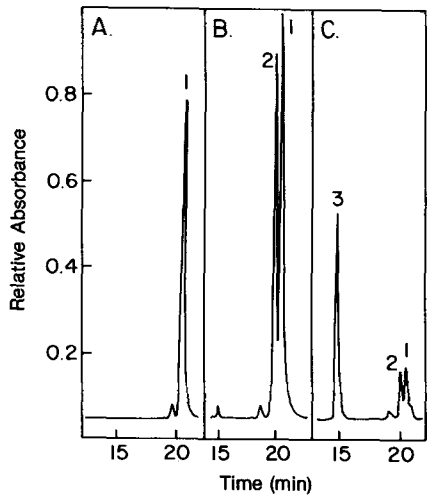

FIG. 2. High-performance liquid chromatography analysis of metabolites formed from 5-phenyl-2,4-pentadienoyl-CoA by 2,4-dienoyl-CoA reductase or by a soluble extract of rat liver mitochondria. A, 5-Phenyl-2,4-pentadienoyl-CoA; B, product formed from 5-phenyl-2,4-pentadienoyl-CoA by partially purified 2,4-dienoyl-CoA reductase in the presence of NADPH; C, metabolites formed when 5-phenyl-2,4-pentadienoyl-CoA was incubated with a soluble extract of rat liver mitochondria in the presence of NADPH. Peak 1, 5-phenyl-2,4-pentadienoyl-CoA; peak 2, 5-phenyl-3-pentenoyl-CoA or 5-phenyl-2-pentenoyl-CoA; peak 3, 5-phenyl-3-hydroxypentanoyl-CoA. For details about the assay, see Experimental Procedures section. Abbreviations as in Figure 1.

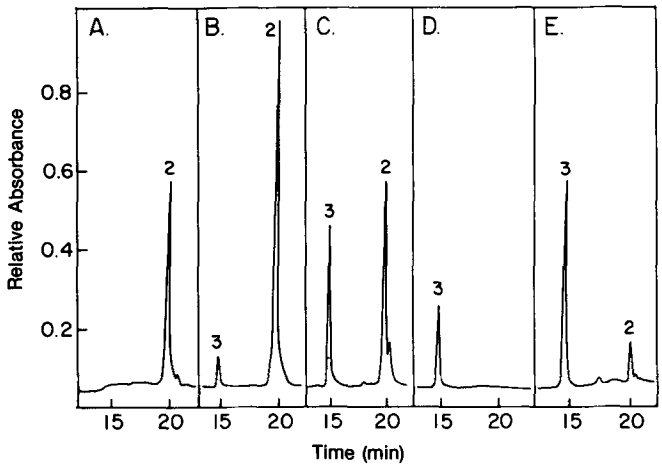

FIG. 3. High-performance liquid chromatography (HPLC) identification of metabolites formed from 5-phenyl-2,4-pentadienoyl-CoA by enzymes of β-oxidation. A, HPLC-purified 5-phenyl-3-pentenoyl-CoA formed from 5-phenyl-2,4-pentadienoyl-CoA by partially purified 2,4-dienoyl-CoA reductase in the presence of NADPH; B, after incubation of 5-phenyl-3-pentenoyl-CoA with purified crotonase (0.25 μg/mL); C, after incubation of 5-phenyl-3-pentenoyl-CoA with peroxisomal trifunctional enzyme (3.5 μg/mL); D, HPLC-purified 5-phenyl-3-hydroxypentanoyl-CoA; E, after incubation of 5-phenyl-3-hydroxypentanoyl-CoA with purified crotonase. Peak 2, 5-phenyl-3-pentenoyl-CoA or 5-phenyl-2-pentenoyl-CoA; peak 3, 5-phenyl-3-hydroxypentanoyl-CoA. Abbreviations as in Figure 1.

decreased in a time-dependent manner (Fig. 1). When partially purified 2,4-dienoyl-CoA reductase was used, only one product was identified by HPLC (Fig. 2B). This compound, which was eluted slightly ahead of the substrate 5-phenyl-2,4-pentadienoyl-CoA (Fig. 2B), was assumed to be 5-phenyl-3-pentenoyl-CoA because both mitochondrial isoforms of the reductase catalyze the reduction of 2,4-dienoyl-CoA to 3-*trans*-enoyl-CoA (1,18). When a soluble extract of rat liver mitochondria served as a source of 2,4-dienoyl-CoA reductase, most of the product was a more polar compound (Fig. 2C). This compound was presumed to be 5-phenyl-3-hydroxypentanoyl-CoA, because 5-phenyl-3-pentenoyl-CoA would be converted to 5-phenyl-3-hydroxypentanoyl-CoA by the sequential actions of Δ^3,Δ^2-enoyl-CoA isomerase and enoyl-CoA hydratase (crotonase) present in the mitochondrial extract. The product formed from 5-phenyl-2,4-pentadienoyl-CoA by partially purified 2,4-dienoyl-CoA reductase was purified by HPLC (Fig. 3A). When this compound, presumably 5-phenyl-3-pentenoyl-CoA, was incubated with enoyl-CoA hydratase (crotonase), most of it remained unchanged except for a small amount that was converted to the more polar compound (Fig. 3B). The formation of the latter compound was most likely due to the presence of a residual amount of Δ^3,Δ^2-enoyl-CoA isomerase in the preparation of purified crotonase. However, when the reduction product was incubated with the peroxisomal trifunctional enzyme, which contains enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and Δ^3,Δ^2-enoyl-CoA isomerase activities (8,9), a significant part of the compound was converted to the polar compound (Fig. 3C). Together, these observations support the conclusion that the product formed by reduction of 5-phenyl-2,4-pentadienoyl-CoA is 5-phenyl-3-pentenoyl-CoA, which cannot be hydrated by enoyl-CoA hydratase. However, if the reduction product is first isomerized to 5-phenyl-2-pentenoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase present in the mitochondrial extract or associated with the trifunctional enzyme, it can be hydrated to 5-phenyl-3-hydroxypentanoyl-CoA by the enoyl-CoA hydratase activity present in the extract or associated with the trifunctional protein. The identity of the polar reaction product, presumed to be 5-phenyl-3-hydroxypentanoyl-CoA, was established by purifying (Fig. 3D) and incubating it with crotonase. Part of the compound was dehydrated to 5-phenyl-2-pentenoyl-CoA, which was eluted from the HPLC column at the same position where 5-phenyl-3-pentenoyl-CoA emerged (compare Fig. 3A and 3E). The relative intensities of peaks 3 and 2 (Fig. 3E) agree with the equilibrium ratio of 3.2 reported for hydrated vs. dehydrated forms of 2-enoyl-CoA (19).

The above characterization of products establishes that the NADPH-dependent reduction of 5-phenyl-2,4-pentadienoyl-CoA (Fig. 4, compound **1**) by 2,4-dienoyl-CoA reductase present in a soluble extract of rat liver mitochondria initially yields 5-phenyl-3-pentenoyl-CoA (Fig. 4, compound **2**). However, as other β-oxidation enzymes are present in the tissue homogenate, 5-phenyl-3-pentenoyl-CoA will be converted to 5-phenyl-2-pentenoyl-CoA (Fig. 4, compound **3**) by Δ^3,Δ^2-enoyl-CoA isomerase, and the latter compound will be partially hydrated by enoyl-CoA hydratase to 5-phenyl-3-hydroxypentanoyl-CoA (Fig. 4, compound **4**). None of the reactions subsequent to

METHOD

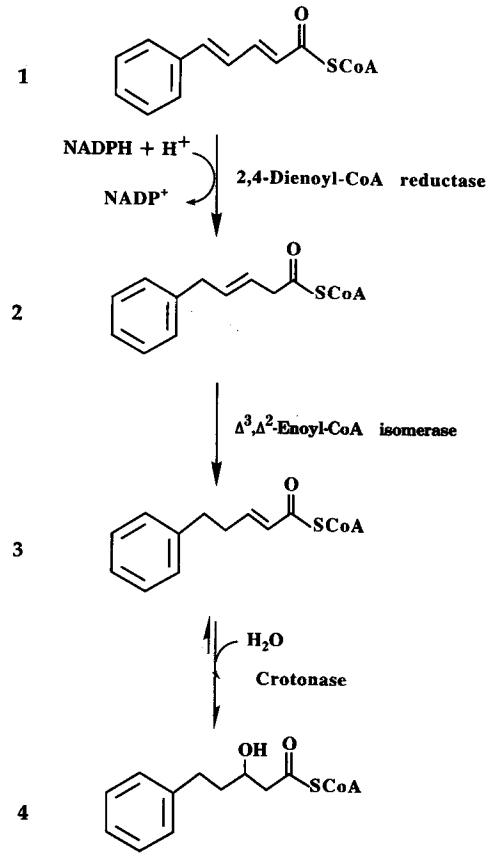

FIG. 4. NADPH-dependent reduction of 5-phenyl-2,4-pentadienoyl-CoA and subsequent β-oxidation reactions catalyzed by a soluble extract of rat liver mitochondria. 1, 5-Phenyl-2,4-pentadienoyl-CoA; 2, 5-phenyl-3-pentenoyl-CoA; 3, 5-phenyl-2-pentenoyl-CoA; 4, 5-phenyl-3-hydroxypentanoyl-CoA. Abbreviations as in Figure 1.

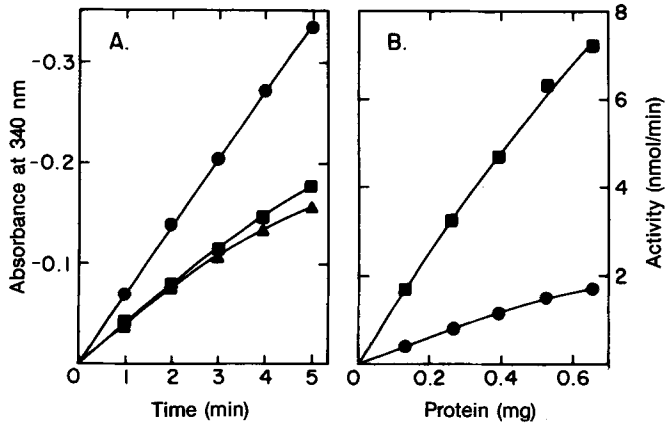

FIG. 5. NADPH-dependent reduction of 5-phenyl-2,4-pentadienoyl-CoA or 2,4-decadienoyl-CoA by 2,4-dienoyl-CoA reductase present in a soluble extract of rat liver mitochondria. A, absorbance change at 340 nm as a function of time. The reaction mixture (1 mL) contained 0.2 M KP_i (pH 8), 0.1 mM NADPH, 0.12 mg of mitochondrial protein and either 25 μM 5-phenyl-2,4-pentadienoyl-CoA (●), or 25 μM 2-*trans*-4-*cis*-decadienoyl-CoA (■), or 25 νM 2-*trans*-4-*trans*-decadienoyl-CoA (▲); B, activity of 2,4-dienoyl-CoA reductase as a function of soluble mitochondrial protein from rat liver, with 2-*trans*-4-*cis*-decadienoyl-CoA (■) or 5-phenyl-2,4-pentadienoyl-CoA as substrate (●). Assay conditions were identical to those given under A. Abbreviations as in Figure 1.

the reduction of 5-phenyl-2,4-pentadienoyl-CoA are contributing to the absorbance change at 340 nm. If NAD^+ were present in the incubation mixture, 5-phenyl-3-hydroxypentanoyl-CoA might be oxidized by 3-hydroxyacyl-CoA dehydrogenase to yield NADH, and thereby give rise to an increase in the absorbance at 340 nm.

When 2,4-dienoyl-CoA reductase, present in a soluble extract from rat liver mitochondria, was assayed with 5-phenyl-2,4-pentadienoyl-CoA and for comparison with 2-*trans*-4-*trans*-decadienoyl-CoA or 2-*trans*-4-*cis*-decadienoyl-CoA as substrates, the results shown in Figure 5 were obtained. With 5-phenyl-2,4-pentadienoyl-CoA as substrate, the change in absorbance at 340 nm as a function of time was linear for at least 5 min. By contrast, the progress curves obtained with the other two substrates deviated from linearity almost immediately after the reaction was initiated. Consequently, reliable values for initial velocities could not be obtained with 2,4-decadienoyl-CoA thioesters as substrates. The decline in rates seen toward the end of the reaction (Fig. 5A) was due to substrate depletion. The greater linearity of the novel assay is partly due to the greater absorptivity of the substrate. Thus, the amount of 5-phenyl-2,4-pentadienoyl-CoA that must be converted to product to obtain a certain absorbance change is only one-eighth of the amount of 2,4-decadienoyl-CoA. This situation greatly reduces the likelihood that nonlinear progress curves will be obtained with the modified assay method. Another advantage of the novel assay procedure is that under identical conditions the absorbance change with 5-phenyl-2,4-pentadienoyl-CoA as substrate is twice the change obtained with either of the isomeric 2,4-decadienoyl-CoA thioesters (Fig. 5A). However, when reductase activities are calculated from absorbance changes, a different picture is obtained. As shown in Figure 5B, the reductase activity with 2-*trans*-4-*cis*-decadienoyl-CoA as substrate is almost four times higher than with 5-phenyl-2,4-pentadienoyl-CoA. The apparent difference between the results shown in Figure 5 (A and B) is due to different extinction coefficients at 340 nm associated with the reduction of 5-phenyl-2,4-pentadienoyl-CoA by NADPH (ε = 50,500 M^{-1} cm^{-1}) and of 2,4-decadienoyl-CoA by NADPH (ε = 6,220 M^{-1} cm^{-1}). What is crucial, when the sensitivity of the assay method is considered, is the greater absorbance change with 5-phenyl-2,4-pentadienoyl-CoA as a substrate as compared to the conventional assay with either isomer of 2,4-decadienoyl-CoA. Kinetic parameters (K_m, V_{max}) were determined for the reduction of 5-phenyl-2,4-pentadienoyl-CoA and 2-*trans*-4-*cis*-decadienoyl-CoA by 2,4-dienoyl-CoA reductase at a fixed concentration of 0.1 mM NADPH. The apparent K_m values for 5-phenyl-2,4-pentadienoyl-CoA (9.5 μM) and 2-*trans*-4-*cis*-decadienoyl-CoA (9.1 μM) were virtually identical, whereas the maximal velocity with the new substrate was eight times slower (0.1 U/mg) than the V_{max} (0.8 U/mg) of the conventional assay.

The modified spectrophotometric assay of 2,4-dienoyl-CoA reductase described in this report represents an im-

provement over the currently used method due to the greater absorbance change that remains linear for a longer period of time. As 5-phenyl-2,4-pentadienoyl-CoA and 2,4-decadienoyl-CoA are prepared by two-step syntheses of equal difficulty, the modified method is recommended for routine assays of 2,4-dienoyl-CoA reductase.

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COMMUNICATION

Positional Distribution of Octadecadienoic Acids in Sponge Phosphatidylethanolamines

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The distribution of C14–C22 fatty acids in the phosphatidylethanolamines isolated from the sponges *Agelas* sp. and *Spongia tampa* was investigated. Selective changes with phospholipase A_2 (from *Agkistrodon halys blomhoffii*) followed by thin-layer chromatographic separation of the resulting lysophosphatidylethanolamines and free fatty acids and subsequent methylation with HCl/MeOH and diazomethane, respectively, revealed that the 5,9-octadecadienoic acid and the 9,12-octadecadienoic acid present showed no preference for either position *sn*-1 or *sn*-2 in these phosphatidylethanolamines. The other saturated and unsaturated fatty acids with chains between 14 and 22 carbons long were also found to be equally distributed between positions *sn*-1 and *sn*-2 in the phosphatidylethanolamines in these sponges. The results contrast with what is known about the distribution in most mammalian phospholipids, such as the phosphatidylcholines from human erythrocytes, where octadecadienoic acid typically occupies the *sn*-2 position.

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There is little information available on the positional distribution of fatty acids (FA) in the phospholipids of marine organisms (1). However, some interesting species differences in the distribution of individual phospholipid FA have been reported. For example, docosahexaenoic acid has been shown to exclusively occupy the *sn*-2 position in the phospholipids from whale and lobster (1), while in phosphatidylcholines (PC) from muscle of salmon and tuna, more than 40% of this acid was found at the *sn*-1 position (2). Oleic acid has also been found in some species of whale to occupy the *sn*-1 position in PC whereas in cod and scallop muscle, oleic acid seems to prefer the *sn*-2 position (2). In fish and other animal PC, 9,12-octadecadienoic acid (18:2) is typically found at the *sn*-2 position (1).

Sponge phospholipids with very long-chain FA (C_{26}–C_{30}) have a random FA distribution between *sn*-1 and *sn*-2 (3). For example, 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphoethanolamine was isolated from the sponges *Microciona prolifera* and *Parasperella psila* and characterized by fast-atom bombardment and tandem mass spectrometry (4). The sponge *Aplysina fistularis* was found to contain 1,2-di-5,9,23-triacontatrienoyl-*sn*-glycero-3-phosphoethanol-amine (5). Phospholipids with polyunsaturated FA (e.g., 20:4 and 22:6) have also been found in sponges. For example, the sponge *Higginsia tethyoides* contains 1,2-di-5,8,11,14-docosatetraenoyl-*sn*-glycero-3-phosphocholine (5), whereas *M. prolifera* synthesizes 1,2-di-(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)docosahexaenoyl-*sn*-glycero-3-phosphocholine as the major PC molecular species (4). Phospholipid molecular species found in higher organisms, such as those found in human erythrocytes, possess a more ordered FA distribution (6). Thus, saturated FA are normally found at the 1-position, and unsaturated FA normally occupy the 2-position (6,7).

The positional distribution of octadecadienoic acids, including linoleic acid, in phospholipids from marine sponges has not been studied previously. In the present work, we determined the positional distribution of two octadecadienoic acids, namely 5,9-octadecadienoic acid and 9,12-octadecadienoic acid, in phosphatidylethanolamines (PE) isolated from the sponges *Agelas* sp. and *Spongia tampa*.

MATERIALS AND METHODS

Agelas sp. was collected August 1, 1992, near Mona Island, Puerto Rico, at a depth of 25 m. *Spongia tampa* was collected in July 1992 at a depth of 1 ft from the mangroves of La Parguera, Puerto Rico. The sponges were freeze-dried in a Labconco Freeze Dryer 4.5 (Model 77510; Labconco, Kansas City, MO). The sponges (30–50 g) were then carefully cleaned of all nonsponge debris and cut into small pieces. Extraction with 250 mL of chloroform/methanol (1:1, vol/vol) yielded the total lipids. The neutral lipids, glycolipids and phospholipids (20 mg) were separated by column chromatography on silica gel (60–200 mesh) using the procedure of Privett *et al.* (8). The phospholipid classes were fractionated by thin-layer chromatography (TLC) on silica gel 60 using chloroform/methanol/NH_4OH (65:35:5, by vol) as solvent. Spray reagents such as molybdenum blue (phospholipids) and ninhydrin (amino groups) were used for the identification of the phospholipids. PE was isolated by preparative TLC under the conditions described above. The TLC fraction identified as PE was scraped from the plate, and the phospholipids were extracted from the silica gel with $CHCl_3$/MeOH/H_2O (50:45:5, by vol).

PC from human erythrocytes was isolated as follows. Duplicate samples of 1.0 mL of a 25% cell suspension in tris [tris(hydroxymethyl)aminomethane] buffered saline (1.2 μmol total phospholipid) were extracted according to Folch *et al.* (9). The total extract was applied to a 5 × 20 cm Whatman pre-coated silica gel plate and developed with $CHCl_3$/MeOH/H_2O (64:32:5, by vol), which contained 0.01% butylated hydroxytoluene as antioxidant. Lipid was visualized with 2′,7′-dichlorofluorescein, and the band corresponding to PC was scraped off.

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Abbreviations: FA, fatty acid(s); GC/MS, gas chromatography/mass spectrometry; PC, phosphatidylcholine(s); PE, phosphatidylethanolamine(s); TLC, thin-layer chromatography; tris, tris(hydroxymethyl)aminomethane.

Phospholipids were enzymatically cleaved by dissolving the purified phospholipid fraction (1.2 μmol) in 1.0 mL of diethyl ether/MeOH (95:5, vol/vol) together with 10 μg of neutral phospholipase A_2 (purified from *Agkistrodon halys blomhoffii*) containing 140 mM tris-HCl (pH = 7.4) and 10 mM $CaCl_2$ (10–12). The reaction mixture was thoroughly mixed by vortexing and incubated at room temperature for 10 min. The reaction time was kept short to minimize acyl migration. The lipids were extracted according to Folch *et al.* (9), and the resulting free FA, unreacted phospholipids and lysophospholipids were separated by preparative TLC on silica gel using $CHCl_3$/MeOH/H_2O (65:35:5, by vol) as solvent. The resulting free FA from the *sn*-2 position of the phospholipids were esterified with diazomethane by adding 200 μL of a saturated solution of diazomethane in ether. The ethereal layer was washed with H_2O (2 × 0.5 mL) and dried under a stream of nitrogen. The fatty acyl components of the lysophospholipids were analyzed as methyl esters by reaction with methanolic hydrogen chloride (13) followed by chromatographic purification on a small column and elution with hexane/diethyl ether (9:1, vol/vol). The resulting methyl esters were analyzed by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard 59970 MS ChemStation (Hewlett-Packard, Palo Alto, CA) equipped with a 30 m × 0.32 mm nonpolar fused silica column (Supelco, Bellefonte, PA) with SPB™-1 as the bonded phase.

GC/Fourier transform infrared (FTIR) spectra were recorded on a Nicolet (Madison, WI) 740 FTIR spectrometer. The double bonds of the monoenoic and dienoic methyl esters were localized by preparing the corresponding dimethyl disulfide derivatives by dissolving the esters (2 mg) in dimethyl disulfide (0.2 mL), adding a solution (0.05 mL) of iodine in diethyl ether (60 mg/mL), and heating the solution at 50°C for 24 h as described previously (14).

RESULTS AND DISCUSSION

The total phospholipid FA composition of the sponge *Agelas* sp. was previously reported (15), and a series of novel brominated FA from the phospholipids of this sponge was isolated. The total phospholipid FA from *S. tampa* have not been reported previously. In the present study, we identified the 5,9-pentacosadienoic acid and 5,9-hexacosadienoic acid as the more prominent phospholipid FA of *S. tampa*. We also identified, in trace amounts, the 9- and 16-pentacosenoic acids as well as the 9-, 15- and 16-hexacosenoic acids, which had previously been found in sponge phospholipids (3). We were able to isolate (by TLC) a pure PE fraction, which contained C_{14}–C_{22} FAs and was free of contaminants. PC was only found in minor amounts in the sponges, and we were not able to isolate a pure PC fraction that was free of other lipids.

The purified PE, from both *Agelas* sp. and *S. tampa*, afforded C_{14}–C_{22} FA after MeOH/HCl transesterification. The principal saturated FA in the isolated PE were tetradecanoic acid (14:0), hexadecanoic acid (16:0) and

TABLE 1

Positional Distribution of Fatty Acids in Phospholipids from *Agelas* sp. and *Spongia tampa*[a]

	Distribution (wt%)					
	Agelas sp.		*S. tampa*		Control[b]	
Fatty acid	*sn*-1	*sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
Tetradecanoic (14:0)	5	3	3	6	—	—
13-Methyltetradecanoic (*i*-15:0)[c]	1	1	1	1	—	—
12-Methyltetradecanoic (*ai*-15:0)[c]	1	1	1	1	—	—
Pentadecanoic (15:0)	2	2	1	3	—	—
9-Hexadecenoic (16:1)	2	3	2	4	0	0.4
Hexadecanoic (16:0	20	14	20	23	53	5
15-Methylhexadecanoic (*i*-17:0)	1	1	1	1	—	—
14-Methylhexadecanoic (*ai*-17:0)	2	1	1	1	—	—
Heptadecanoic (17:0)	1	1	1	2	—	—
5,9-Octadecadienoic (18:2)	4	4	—	—	—	—
9,12-Octadecadienoic (18:2)	—	—	5	4	0.5	32
9-Octadecenoic (18:1)	32	38	34	28	0.5	29
11-Octadecenoic (18:1)	4	7	7	4	—	—
Octadecanoic (18:0)	13	11	8	12	46	0.3
13-Docosenoic (22:1)	12	13	15	10	—	—

[a]The experimental error was calculated to be about 1%. No alkyl ethers or alkenyl ethers were identified in these phospholipids.

[b]The data were obtained on phosphatidylcholines from human erythrocytes when analyzed under the same conditions as the sponge phosphatidylethanolamines. Other fatty acids exclusively identified at the *sn*-2 position of the phosphatidylcholines from human erythrocytes were: 20:3 (4.2%), 20:4ω6 (21%), 22:4ω6 (2.2%), 22:5ω6 (0.9%), 22:5ω3 (0.9%) and 22:6ω3 (3.8%). No polyunsaturated fatty acids were detected at the *sn*-1 position.

[c]*i, iso; ai, anteiso.*

octadecenoic acid (18:0). These acids were identified by GC/MS and by GC co-injection with authentic standards (Table 1). The saturated FA accounted for 28–41% of the total FA in PE from both sponges. The principal FA in the PE were monounsaturated acids of the n-9 family, i.e., 9-octadecenoic acid (18:1Δ9) and 13-docosenoic acid (22:1Δ13), which together accounted for more than half of the total acids in this phospholipid (38–51%). Other monounsaturated FA belonging to the n-7 family were also found, namely 9-hexadecenoic acid (16:1Δ9) and 11-octadecenoic acid (18:1Δ11), which accounted for 6–10% of the total FA observed. The only octadecadienoic acid in *Agelas* sp. was characterized as 5,9-octadecadienoic acid (18:2Δ5,9) by MS of its methyl ester and dimethyl disulfide derivative, and based on GC retention times (4). On the other hand, the sponge *S. tampa* contained the 9,12-octadecadienoic acid as the only dienoic acid in the isolated PE.

The positional distribution of the C_{14}–C_{22} FA in the PE from *Agelas* sp. and *S. tampa* was examined by means of enzymatic digestion with neutral phospholipase A_2 from *A. halys blomhoffii*, which releases FA from the *sn*-2 position (10–12). The purified enzyme was obtained from *A. halys blomhoffii* as previously reported (12), and it showed a single band on sodium dodecyl sulfate gel electrophoresis, excluding contamination with lipase or phospholipase A_1 (12). The FA composition at the *sn*-1 and *sn*-2 positions, as determined by this method, is presented in Table 1. It is apparent that the FA distribution between *sn*-1 and *sn*-2 is quite similar. For example, the saturated FA 14:0, 16:0 and 18:0 were equally found at positions *sn*-1 and *sn*-2, with a slight preference for position *sn*-1 in both sponges. The typical odd-chain branched FA *iso*- and *anteiso*-15:0 and 17:0 were also found to occupy both positions, with no clear preference for *sn*-1 or *sn*-2. The most abundant FA in the mixture, namely 18:1Δ9 and 22:1Δ13, were also found both at positions *sn*-1 and *sn*-2, but a slight preference for *sn*-2 was observed in *Agelas* sp. Thus, in these sponges saturated and monounsaturated FA can equally occupy the *sn*-1 and *sn*-2 positions in PE. Such lack of positional specificity has been observed before in several bacteria (16,17).

The lysoPE and free FA formed after PLA_2 hydrolysis in the present study were produced in an approximately 1:1 molar ratio. We estimated that the extent of hydrolysis was *ca.* 60%. The FA compositions of the PE before hydrolysis and of the residual PE after hydrolysis were shown to be similar. As a control, we also applied the methodology we used to PC isolated from human erythrocytes. We selected these phospholipids because the positional distribution of FA, and specifically that of the 9,12-octadecadienoic acid, has been thoroughly studied (6). As can be seen from Table 1, no significant 1,2-acyl migration occurred in the method we used.

One interesting finding in these sponges was the lack of any positional specificity for the octadecadienoic acids, i.e., for example in *Agelas* sp. the 5,9-octadecadienoic acid was equally found at positions *sn*-1 and *sn*-2 in the PE. This may imply the presence of the identically substituted 1,2-di-(5*Z*,9*Z*)-5,9-octadecadienoyl-*sn*-glycero-3-phosphoethanolamine, similar to what has been reported for the longer chain "demospongic" FA of sponges (3). In *S. tampa*, a similar observation was made with the methylene-interrupted 9,12-octadecadienoic acid, present both at positions *sn*-1 and *sn*-2 of PE.

This study represents the first effort to elucidate the positional distribution of C_{14}–C_{22} FA in the phospholipids of sponges. The PE studied have FA similar to cyanobacteria (blue-green algae) as the 16:1Δ9 acid is considered a major cyanobacterial acid (18). However, the 18:2(Δ5,9) and 18:2(Δ9,12) acids are not common in bacteria (18). In particular, the 18:2(Δ5,9) acid has not been found in bacteria. Thus, the PE we isolated could originate from microbial symbionts and sponge cells. It is also interesting to note that the two sponges collected from two different habitats have a very similar PE FA distribution.

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Production of Docosahexaenoic Acid by Marine Bacteria Isolated from Deep Sea Fish

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Five bacterial strains isolated from the intestine of deep sea fish were shown to produce docosahexaenoic acid (22:6n-3; DHA) at a level of 6.4 to 11.6% of total fatty acids when incubated in DHA-free medium. In all of the strains examined, other polyunsaturated fatty acids were barely detectable, except for eicosapentaenoic acid (20:5n-3). A typical strain, such as T3615, produced DHA at a concentration of about 0.8 mg/L within six days of aerobic incubation at 5°C and under atmospheric pressure. The T3615 strain, belonging to the genus *Vibrio*, is rod-shaped, Gram-negative, motile and facultatively anaerobic.

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It is now commonly believed that docosahexaenoic acid (22:6n-3; DHA) has some beneficial health effects. Hence, DHA has attracted considerable scientific, as well as public, attention (1,2). DHA is also known to be essential for the normal growth of fish at the larval stage (3). DHA concentrates are currently produced from fish oils. However, it is still difficult to obtain highly pure DHA preparations, which is partly due to the fact that the DHA content of fish oils fluctuates widely depending on the species of fish used, as well as the season.

Other sources of DHA are being sought among algae and fungi (4,5). Bacterial production of DHA would be of interest because of the ease with which bacteria can be cultured in modern biotechnology equipment. Only one study dealing with the bacterial production of DHA has been published so far (6), although there are some papers describing bacteria that can produce other polyunsaturated fatty acids, such as eicosapentaenoic acid (20:5n-3; EPA) (7–10). We now found that out of nine bacterial strains isolated from the intestine of deep sea fish, five produced DHA.

MATERIALS AND METHODS

Isolation and cultivation of microorganisms. The bacterial strains were isolated from the intestines of two species of deep sea fish, *Histiobranchus* sp. and *Coryphaenoides yaquinae,* which were caught at depths of 3,200–5,300 m during crab-pot fishing. Diluted intestinal contents were spread on marine agar 2216 (Difco Laboratories, Detroit, MI) plates and incubated at 5°C for two months. Colonies were purified by being streaked in a series of three transfers onto the plates. Nine strains isolated in this manner were further analyzed, lipids were recovered and the fatty acid composition was determined. The strain numbers, species names of the fish specimens and the depths at which the fish were caught are given in Table 1. Bacterial growth was monitored by counting 4,6-diamidino-2-phenylindole-stained cells by epifluorescence microscopy. Strains were classified by taxonomic tests following an established identification scheme (11).

TABLE 1

Strains of Bacteria and the Fish Species from Which the Bacteria Were Isolated

Strain number	Fish number	Species name of fish	Depth (m)[a]
T3602	36	*Histiobranchus* sp.	4,700
T3615	36	*Histiobranchus* sp.	4,700
T4607	46	*Coryphaenoides yaquinae*	4,300
T4609	46	*C. yaquinae*	4,300
T4714	47	*C. yaquinae*	3,200
T4715	47	*C. yaquinae*	3,200
T5301	53	*C. yaquinae*	5,300
T5603	56	*C. yaquinae*	5,200
T5611	56	*C. yaquinae*	5,200

[a]The depth at which the fish were caught.

Lipid analyses. The strains were grown in marine broth 2216 (Difco Laboratories) at 5°C and under atmospheric pressure while shaking. Cells were harvested by centrifugation, and the lipids were extracted according to the procedure of Folch *et al.* (12). Extraction of the same medium before use served as control. Fatty acids were transmethylated in 2.5% methanolic HCl by heating at 85°C for 3 h. The fatty acid methyl esters were analyzed by gas chromatography using an Omegawax 250 capillary column (25 m × 0.25 mm i.d.; Supelco, Bellefonte, PA) and helium as carrier gas. The oven temperature was 180°C. The samples were further analyzed using a SP-2330 capillary column (30 m × 0.25 mm i.d.; Supelco) with the oven temperature at 150°C. The fatty acid methyl esters were identified by comparison with known standards (Sigma, St. Louis, MO). Tricosanoic acid was used as internal standard. The fatty acid methyl esters were also confirmed by gas chromatography/mass spectrometry using a JMS-DX303 instrument (JEOL, Tokyo, Japan) with an Omegawax 320 column (30 m × 0.32 mm i.d.). The oven temperature was 200°C. Electron impact mass spectra were measured at 70 eV.

RESULTS AND DISCUSSION

The fatty acid compositions of nine of the strains examined are given in Table 2. Five strains out of the nine strains examined produced DHA. The DHA produced by the strains accounted for 6.4 to 11.6% of the total fatty acids when incubated in marine broth 2216. Small amounts (<0.01 mg/L) of palmitic acid (16:0), palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) were present in the medium before use. Polyunsaturated fatty acids, such as

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

TABLE 2

Fatty Acid Compositions of Cultured Bacteria Originally Isolated from Intestinal Contents of Deep Sea Fish

Fatty acids	Strain number of fish				
	T3615	T4714	T4715	T5603	T5611
14:0	17.8	6.9	9.0	16.8	15.8
14:1n-5	12.2	2.0	2.4	4.2	5.2
15:0	trace[a]	0.5	0.6	1.0	0.8
16:0	10.7	16.8	16.9	15.7	16.4
16:1n-7	45.3	53.4	56.9	48.3	49.8
18:0	trace	0.5	0.5	trace	trace
18:1n-9	trace	1.2	0.9	trace	trace
18:1n-7	0.6	2.8	2.2	trace	trace
18:3n-6	trace	nd	nd	0.9	0.6
20:3n-3	nd[b]	trace	trace	nd	nd
20:4n-3	0.6	0.8	0.5	trace	trace
20:5n-3	1.1	2.7	1.9	2.5	2.6
22:6n-3	9.4	11.6	8.1	9.4	6.4

[a]Trace, <0.5%. [b]nd, Not detectable.

EPA and DHA, were not detected (not listed in Table 2). The results indicated that the bacterial strains synthesized DHA *de novo*. The other major fatty acids in the five strains were myristic acid (14:0), myristoleic acid (14:1n-5), palmitic acid and palmitoleic acid. EPA was also detected in all strains, although DHA-producing bacteria were not shown to produce EPA in an earlier study (6). Other polyunsaturated fatty acids were barely detectable. This simple fatty acid composition would suggest that the purification of DHA from bacterial lipids should be much easier than from other DHA sources, including fish oils or fungal lipids.

The growth curve for a typical strain, T3615, is shown in Figure 1. The cell count reached a maximum of 1.5×10^8 cells/mL after six days during aerobic incubation at 5°C and under atmospheric pressure while shaking. Under these conditions, 122 mg of dry cells and 11.9 mg of total lipids per L of culture were obtained. The DHA yield was 0.8 mg/L. The strain was shown to be barophilic as it grew to 1.2×10^8 cells/mL after three days at 5°C at higher pressure (41.4 MPa). The strain also produced DHA under high pressure, but a quantitative comparison of DHA yields obtained at atmospheric pressure and at high pressure is different because other factors affecting growth, such as the supply of oxygen, cannot be kept constant.

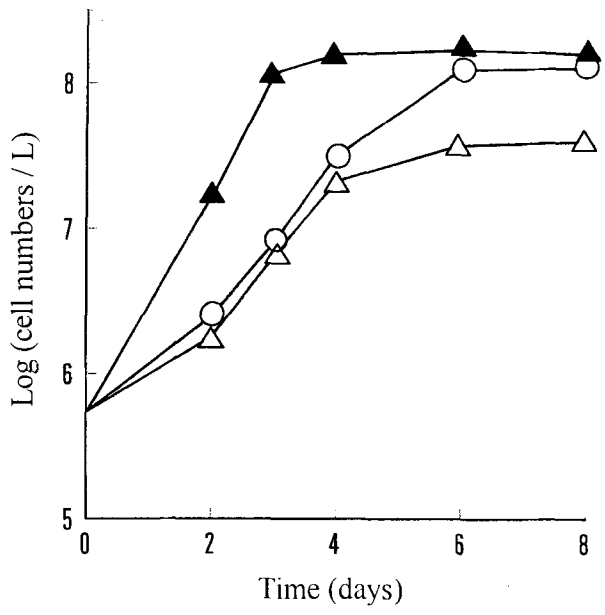

FIG. 1. Growth curves of the strain T3615 at 5°C under atmospheric pressure and at 41.4 Mpa. ○, Atmospheric pressure with shaking; △, atmospheric pressure without shaking; and ▲, 41.4 Mpa without shaking.

Cells of the strain T3615 were 1–2 μm in diameter and 2–3 μm in length, although the size was somewhat dependent on culture conditions. The strain is rod-shaped, motile and Gram-negative, and not luminous. It produced acids but not gas from glucose, and required sea water or artificial sea water for growth. These characteristics indicate that the strain belongs to the genus *Vibrio*.

The results suggest that bacteria may become useful for the production of DHA, although the productivity of the strains studied here seems lower than that of algae or fungi (4, 5). EPA synthesis has already been reported for *Escherichia coli* that carry part of the DNA of EPA-producing bacteria (13). As the cultivation of *E. coli* is much easier and requires less energy, bacterial DHA production could be improved by using biotechnological approaches and gene manipulation and, for example, DNA of the isolates described in this study.

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ARTICLE

Distribution of Cholesterol Among Its Carriers in the Bile of Male and Female Hamsters

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The distribution of cholesterol among its carriers was studied in the bile of male and female hamsters. Sasco hamsters (Sasco Inc., Omaha, NE) were fed a semipurified diet with 0.0% cholesterol and 4% butterfat (group 1, males; group 4, females); a semipurified diet with 0.3% cholesterol and 1.2% palmitic acid (group 2, males; group 5, females); and a semipurified diet with 0.3% cholesterol and 4% safflower oil (group 3, males; group 6, females). At the end of six weeks, gallstones were found only in male hamsters receiving both cholesterol and dietary fat (fatty acid) (incidence of cholesterol stones: 90% in group 2; 22% in group 3). The biliary cholesterol carriers were separated and isolated from the bile of the hamsters by gel filtration chromatography, using the method of Pattinson [Pattinson, N.R., Willis, K.E., and Frampton, C.M. (1991) *J. Lipid Res. 32*, 205–214]. In those male hamsters that formed cholesterol gallstones, significant amounts of cholesterol were present in the void volume which contained large cholesterol phospholipid vesicles (void volume vesicles) (23% in group 2 and 15% in group 3). Smaller cholesterol/phospholipid vesicles were eluted next (fractions 30–45) and contained 15% of biliary cholesterol in group 2 and 21% in group 3. The remainder of the cholesterol was associated with mixed cholesterol/phospholipid/bile salt micelles. The cholesterol/phospholipid ratio was larger in both the void volume vesicles and small vesicles (2.40 and 1.48 in group 2; 2.56 and 1.33 in group 3, respectively) compared to the micelles (about 0.3 in groups 2 and 3). In contrast, the bile of the female hamsters contained few vesicles (3% small vesicles in group 5) and the cholesterol/phospholipid ratio of these vesicles was lower (0.94). Hamsters fed cholesterol-free diets (groups 1 and 4) had no biliary cholesterol/phospholipid vesicles; all cholesterol was present in micelles. The results suggest that both the gender and the diet of the hamsters affected the distribution of biliary cholesterol between vesicles and micelles. The development of cholelithiasis in this animal model appears to depend on the rapid nucleation of cholesterol-rich phospholipid vesicles in bile.

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Cholesterol/phospholipid vesicles and cholesterol/phospholipid/bile salt mixed micelles are the main cholesterol carriers in gallbladder bile (1–3). It has recently become apparent that the vesicles play an important role in cholesterol gallstone formation (4–8). Nucleation of cholesterol, the initial step in cholelithiasis, is a consequence of vesicular aggregation, and rapid cholesterol nucleation is associated with high cholesterol/phospholipid ratios of vesicles (4,7–9).

A hamster model of cholesterol cholelithiasis has been in use in our laboratory for a number of years (10–16). Lithogenesis in this model is a function of several factors, particularly of dietary constituents and source, age and sex of the experimental animals (11,14–16). Recently, we reported that under standard conditions (lithogenic diet fed for six weeks) gallstones were formed in male hamsters but not in female hamsters of either the Sasco (Sasco Inc., Omaha, NE) or Charles River (Charles River Laboratories, Wilmington, MA) "strains" (16). We hypothesized that lithogenesis might occur more rapidly in male hamsters as compared to female hamsters after a six-week feeding if (i) the bile of the former contains biliary cholesterol in the form of vesicles, and (ii) in the males, the vesicular cholesterol/phospholipid ratio is relatively higher than in females. To test this hypothesis, we studied the effect of the gender of the experimental animals on gallstone incidence, the distribution of biliary cholesterol between vesicles and micelles and the vesicular cholesterol/phospholipid ratio in bile.

As observed previously, only the male hamsters developed gallstones in response to the standard lithogenic diet. In these animals, a relatively large amount of cholesterol was carried in void volume vesicles with high cholesterol/phospholipid ratios. These results suggest that the observed sex difference in our hamster model of cholesterol cholelithiasis is due, at least in part, to differences between cholesterol carriers in bile.

MATERIALS AND METHODS

Animals. Male and female golden Syrian hamsters (28 males and 32 females) were obtained from Sasco Inc. Male and female animals were each divided into three groups of nine to eleven animals; their weights averaged 75 g (average age, six weeks). The basal, semipurified diet (SPD) purchased from Dyets, Inc. (Bethlehem, PA) contained 43.7% corn starch, 20% casein, 14.6% dyetrose (soluble starch), 10% fiber (cellulose), 5% salt mix (modified U.S.P. XIV salt mix, no. 200951), 2% corn oil, 0.5% vitamin mix (no. 300000) and 0.2% choline chloride. To this diet was added 4% butterfat (group 1, males; group 4, females); 0.3% cholesterol, 1.2% palmitic acid and 2.5% cornstarch (group 2, males; group 5, females); or 0.3% cholesterol and 4% safflower oil less 0.3% cornstarch (group 3, males; group 6, females). These diets were fed for a period of six weeks. An additional 25 animals (8–9 animals/group) were fed the SPD with 0.3% cholesterol and 1.2% palmitic acid (for two weeks) in order to obtain sufficient bile to

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Abbreviations: CDCA, chenodeoxycholic acid; CSI, cholesterol saturation indices; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; SPD, semipurified diet.

check the reproducibility of the gel filtration technique (groups A–C).

At the end of the experimental period, the hamsters in each group were fasted for 24 h and anesthetized with 20 mg of ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). The gallbladder was examined for the presence of gallstones, and bile was removed with a 100-μL Hamilton syringe and examined by polarized light microscopy (Olympus MCHAP microscope; Olympus Corp., Lake Success, NY) for the presence of cholesterol stones or cholesterol crystals. The remaining fresh bile of each group was combined and centrifuged in 1.5-mL microfuge tubes using an Eppendorf 5415C centrifuge (Brinkmann Instruments, Westbury, NY) at 2000 rpm for 30 min. The fresh supernatant solution was used immediately for separation of the vesicles and micelles by gel filtration chromatography.

Column chromatography. A toluene solution of 0.25 μCi [1α,2α(n)-^{3}H]cholesterol (45.6 Ci/mmol) and a chloroform/methanol (1:1, vol/vol) solution of 0.15 μCi 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (53 mCi/mmol) (Amersham Life Science, Arlington Heights, IL) were mixed and evaporated under N_2. The bile samples were added to the labeled compounds and incubated for 1 h at 37°C with shaking. The labeled bile samples (200 μL) were applied to Sepharose CL-4B-200 (Sigma Chemical Co., St. Louis, MO) columns (20 cm × 1.5 cm i.d.) and eluted with saline buffered with 0.01 M phosphate (pH 7.4) containing 0.04% sodium azide and 6 mM sodium taurocholate as described by Pattinson *et al.* (17); the flow rate was 15.5 mL/h. Fractions of 500 μL each were collected, and an aliquot (100 μL) of each fraction was counted in 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.

Chemical analyses. After hydrolysis, bile acids were determined as the methyl ester acetates by capillary gas–liquid chromatography as described previously (18). Biliary phospholipids were determined enzymatically using the Wako phospholipid B kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Biliary cholesterol was determined as the trimethylsilyl ether by capillary gas–liquid chromatography (11). The cholesterol saturation indices (CSI) of the bile were calculated as reported previously (19,20).

After gel filtration chromatography, the percentage of cholesterol and phospholipid in vesicles and micelles was calculated by determining the total radioactivity of the area under each peak (shown later in Figs. 1 and 2). The cholesterol/phospholipid ratio of vesicles and micelles was calculated using the above percentages.

Statistics. The data for the distribution of cholesterol among vesicles and micelles in animals maintained with SPD, containing 0.3% cholesterol and 1.2% palmitic acid for two weeks, were obtained in triplicate and are summarized as the mean ± SD (Table 5 later in text). Differences in gallstone incidence between groups were determined by chi square.

RESULTS

All hamsters remained healthy during the six-week feeding period, and the animals in all six groups gained similar amounts of weight. The incidence of cholesterol gallstones and biliary cholesterol crystals is shown in Table 1. The groups (1 and 4) receiving the cholesterol-free diet had no gallstones. Male hamsters maintained with 0.3% cholesterol plus 1.2% palmitic acid (group 2) had a 90% incidence of gallstones; those receiving 0.3% cholesterol plus 4.0% safflower oil (group 3) had an incidence of only 22%. In contrast, female hamsters fed the identical diets (groups 5 and 6) did not form gallstones or biliary cholesterol crystals. None of the animals in any group had pigment stones.

Molar proportions of biliary lipids, CSI and cholesterol/phospholipid ratios in bile are summarized in Table 2. In the cholesterol-fed groups, the male hamsters had higher CSI and cholesterol/phospholipid ratios than the females (groups 2 and 3 vs. groups 5 and 6); the CSI of stone-forming groups 2 and 3 was greater than unity—1.27 in group 2 and 1.13 in group 3. The total lipid concentrations of the six groups were similar.

The biliary bile acid composition of the six experimental groups is shown in Table 3. As observed previously (11), cholesterol feeding increased the chenodeoxycholic acid (CDCA)/cholic acid ratio in both male and female hamsters. The biles of male animals contained higher proportions of hyodeoxycholic acid and lower proportions of lithocholic acid and deoxycholic acid as compared with the corresponding groups of female animals.

Figure 1 illustrates the elution patterns of the combined biles of each of the six groups as determined by gel filtration chromatography. In groups 2 and 3, which form cho-

TABLE 1

Incidence of Biliary Cholesterol Gallstones and Biliary Cholesterol Crystals at Sacrifice[a]

Group	Sex	Diet	Cholesterol stones		Cholesterol crystals	
			Number	%	Number	%
1	Male	SPD + 4.0% butterfat + 0.0% cholesterol	0/9	0	0/9	0
2	Male	SPD + 1.2% palmitic acid + 0.3% cholesterol	9/10[b]	90	9/10[b]	90
3	Male	SPD + 4.0% safflower oil + 0.3% cholesterol	2/9	22	2/9	22
4	Female	SPD + 4.0% butterfat + 0.0% cholesterol	0/11	0	0/11	0
5	Female	SPD + 1.2% palmitic acid + 0.3% cholesterol	0/10	0	0/10	0
6	Female	SPD + 4.0% safflower oil + 0.3% cholesterol	0/11	0	0/11	0

[a]See Materials and Methods section for details of the hamster diets. SPD, semipurified diet. Feeding period, six weeks.
[b]Differs significantly from groups 1 and 3–6; $P < 0.005$ by chi square.

TABLE 2

Lipids of Hamster Bile[a]

Group	Sex	Diet	mole %			Total lipid (g/dL)	CSI	Cholesterol/ phospholipid ratio
			Bile acid	Phospholipid	Cholesterol			
1	Male	SPD + 4.0% butterfat + 0.0% cholesterol	90.7	6.9	2.4	11.5	0.67	0.34
2	Male	SPD + 1.2% palmitic acid + 0.3% cholesterol	77.0	15.8	7.2	9.3	1.27	0.46
3	Male	SPD + 4.0% safflower oil + 0.3% cholesterol	84.2	10.8	5.0	10.5	1.13	0.47
4	Female	SPD + 4.0% butterfat + 0.0% cholesterol	91.4	7.3	1.3	8.5	0.37	0.19
5	Female	SPD + 1.2% palmitic acid + 0.3% cholesterol	90.3	7.1	2.6	12.1	0.71	0.36
6	Female	SPD + 4.0% safflower oil + 0.3% cholesterol	88.2	9.2	2.6	9.9	0.66	0.29

[a]See Materials and Methods section for details of the hamster diets. SPD, semipurified diet; CSI, cholesterol saturation index.

lesterol gallstones (see Table 1), significant amounts of cholesterol were present in the void volume vesicles, fractions 20–35, and small vesicles, fractions 35–45. In all groups, a considerable proportion of the labeled cholesterol was eluted in fractions 45–75 (micelles). The percent of cholesterol in the combined vesicular fractions (void volume vesicles and small vesicles) amounted to 38.3% in group 2 and 35.9% in group 3 (Table 4). In groups 1, 4, 5 and 6, the biliary cholesterol was practically all in the micellar fraction, and none of the animals had cholesterol gallstones. The cholesterol/phospholipid ratio of the void volume vesicles and small vesicles in groups 2 and 3 exceeded unity (2.40 and 1.48, group 2; 2.56 and 1.33, group 3) (Table 4). The bile of the female hamsters receiving the lithogenic diet (group 5) appeared to contain some small vesicles (2.9%); the cholesterol/phospholipid ratio of this fraction was less than 1 (0.94). In the bile of animals receiving cholesterol-free diets (groups 1 and 4), cholesterol appeared to be present solely in the form of micelles. The cholesterol/phospholipid ratio of the micelles ranged from 0.17 (group 4) to 0.35 (group 5).

In order to determine the reproducibility of the gel filtration technique, we performed column chromatography in triplicate using the bile of hamsters fed the SPD with 0.3% cholesterol and 1.2% palmitic acid for two weeks. Figure 2 shows the elution profile of the combined bile of these three groups. All groups (A–C) had similar amounts of void volume vesicles and small vesicles in bile and the cholesterol/phospholipid ratio of the vesicles was also similar in these groups (Table 5).

DISCUSSION

Male Sasco hamsters fed an SPD with added 0.3% cholesterol and 1.2% palmitic acid had a high incidence of cholesterol gallstones; cholelithiasis was reduced, but not abolished when 4% safflower oil was substituted for the palmitic acid (14). Female hamsters fed the same diets developed neither biliary cholesterol crystals nor gallstones. No cholesterol gallstones were formed without exogenous cholesterol regardless of whether 4% butterfat or 1.2% palmitic acid were incorporated into the diet (14).

The formation of cholesterol gallstones and crystals is believed to occur from nucleation of vesicles of high cholesterol content (7–9). We hypothesized that in the stone-forming groups vesicles were present in bile. It was necessary to isolate and quantitate the amount of vesicles and micelles to confirm this hypothesis. Consequently, we car-

TABLE 3

Bile Acid Composition of Hamster Bile[a]

Group	Sex	Diet	Bile acid (mg/mL)	LCA (%)	DCA (%)	CDCA (%)	CA (%)	ACA (%)	HDCA (%)	MDCA (%)	Others (%)
1	Male	SPD + 4.0% butterfat + 0.0% cholesterol	82.38	1.54	11.91	17.92	44.97	2.68	12.97	3.83	4.18
2	Male	SPD + 1.2% palmitic acid + 0.3% cholesterol	54.12	1.93	8.66	28.55	44.57	2.10	6.02	1.65	6.52
3	Male	SPD + 4.0% safflower oil + 0.3% cholesterol	68.21	1.99	7.59	33.03	34.30	2.41	12.46	4.08	4.14
4	Female	SPD + 4.0% butterfat + 0.0% cholesterol	61.20	4.57	19.76	14.01	52.85	0.42	1.63	0.98	5.78
5	Female	SPD + 1.2% palmitic acid + 0.3% cholesterol	85.76	4.89	12.65	30.86	38.52	0.25	4.91	2.33	5.59
6	Female	SPD + 4.0% safflower oil + 0.3% cholesterol	68.03	4.42	16.82	25.40	39.34	0.32	3.93	1.84	7.93

[a]See Materials and Methods section for details of hamster diets. LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; ACA, allocholic acid; HDCA, hydeoxycholic acid; MDCA, murideoxycholic acid; SPD, semipurified diet.

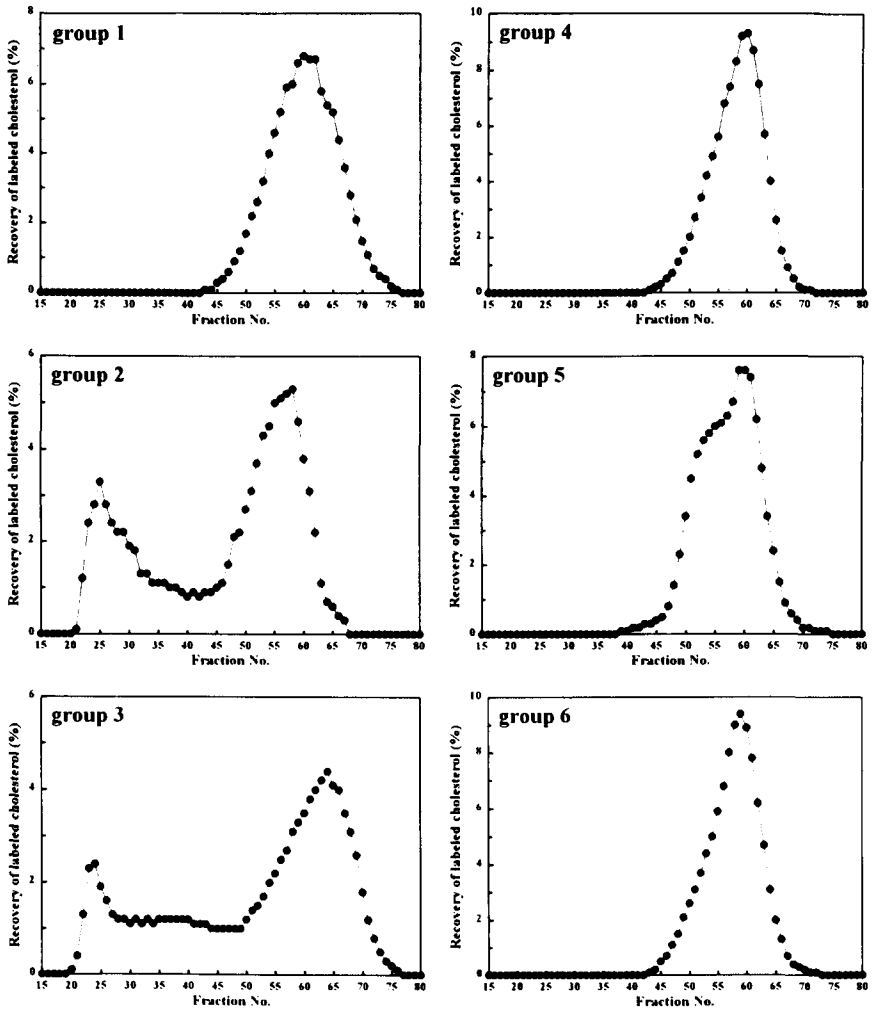

FIG. 1. Gel filtration chromatography of hamster gallbladder bile on Sepharose CL-4B-200 (Sigma Chemical Co., St. Louis, MO). The elution profiles show percent recovery of [^{3}H]cholesterol (●). 1) Male, semipurified diet (SPD) with 0.0% cholesterol and 4% butterfat (group 1); 2) male, SPD with 0.3% cholesterol and 1.2% palmitic acid (group 2); 3) male, SPD with 0.3% cholesterol and 4% safflower oil (group 3); 4) female, SPD with 0.0% cholesterol and 4% butterfat (group 4); 5) female, SPD with 0.3% cholesterol and 1.2% palmitic acid (group 5); 6) female, SPD with 0.3% cholesterol and 4% safflower oil (group 6). The biles of groups 1, 4 and 6 contain micelles only (fractions 45–75). The bile of group 2 contains both void volume vesicles and micelles. The bile of group 3 contains void volume vesicles, small vesicles (fractions 35–45) and micelles. The bile of group 5 contains largely micelles with a small vesicular shoulder. See Table 4 for quantitation.

ried out gel filtration chromatography to obtain these data. The method employed was that of Pattinson *et al.* (17), using Sepharose CL-4B-200. The biles of 9–11 hamsters in each group had to be combined. The small quantities of bile obtained from the individual animals made it necessary to pool bile samples in order to have sufficient material for column chromatography. It would be ideal to carry out column chromatography on individual samples of bile using the exact bile acid concentration and bile acid species in the eluting buffer. However, this is impractical when dealing with the relatively minute quantities of bile available from each hamster (20–50 μL/animal). The use of 6 mM sodium taurocholate, pH 7.4, as the eluting buffer was identical to that of Pattinson *et al.* (17). The choice of 6 mM sodium taurocholate is an estimate based on previous data (17). Because hamster bile and human gallbladder bile are similar (but not identical in composition), we used the same bile acid concentration for our elution buffer (6 mM sodium taurocholate). It would have been ideal to determine the intermicellar bile acid concentration (21), but the small amounts of bile available made this impossible. We verified the reproducibility of our column method using three groups of animals (8–9 animals/group), each fed the SPD containing 0.3% cholesterol and 1.2% palmitic acid for two weeks. The vesicle fractions (%) were very similar in all cases (Table 5 and Fig. 2). It

TABLE 4

Distribution of Cholesterol Among Its Carriers in Hamster Bile[a]

Group	Sex	Diet	Cholesterol (%)			Cholesterol/phospholipid ratio		
			Ves	s-Ves	Mic	Ves	s-Ves	Mic
1	Male	SPD + 4.0% butterfat + 0.0% cholesterol	0	0	100.0	—	—	0.34
2	Male	SPD + 1.2% palmitic acid + 0.3% cholesterol	23.1	15.2	61.7	2.40	1.48	0.31
3	Male	SPD + 4.0% safflower oil + 0.3% cholesterol	14.8	21.1	64.1	2.56	1.33	0.33
4	Female	SPD + 4.0% butterfat + 0.0% cholesterol	0	0	100.0	—	—	0.17
5	Female	SPD + 1.2% palmitic acid + 0.3% cholesterol	0	2.9	97.1	—	0.94	0.35
6	Female	SPD + 4.0% safflower oil + 0.3% cholesterol	0	0	100.0	—	—	0.29

[a]Vesicles and micelles were separated by gel filtration chromatography (Ref. 17). See Materials and Methods section for details. Ves, void-volume vesicles; s-Ves, small vesicles; Mic, Micelles; SPD, semipurified diet.

seems highly plausible that the results of the other gel filtration chromatographic separations (Table 4) would also be reproducible if larger groups of animals were used for each study. However, the cost of each experiment because of the large number of animals required limits our ability to obtain this additional data.

Pattinson *et al.* (17) reported three types of cholesterol carriers in bile, namely, vesicles (eluted in the void volume), small vesicles and mixed micelles. Only gallstone patients, but not the stone-free control subjects, had vesicles in their gallbladder bile. In addition, Schriever and Jüngst (6) also reported that biliary vesicles were present in 18 of 19 gallbladder biles of stone-containing patients but in only 3 of 14 biles of control subjects. In the present study, vesicles were detected in those groups of animals (groups 2 and 3) in which gallstones were formed. Presumably, cholesterol nucleates from vesicles with high cholesterol/phospholipid ratios (7,9). Table 4 shows that the vesicles in groups 2 and 3 had high cholesterol/phospholipid ratios (2.40 and 2.56, respectively). In contrast, in the female animals in groups 5 and 6, no vesicles were detected in the gallbladder bile and no gallstones formed. These results seem to confirm that a relationship between the presence of vesicles and cholelithiasis in these animals may well exist.

Both total biliary lipid concentration and the cholesterol saturation of bile play an important role in modifying the relative concentrations of biliary cholesterol carriers (6,9,22). In the present study, the total biliary lipid concentration of males and females was similar. The cholesterol saturation indices of groups 2 and 3 exceeded unity, and vesicles were present in only these two groups. Although stone incidence in groups 2 and 3 differed, the bile of both groups contained considerable proportions of biliary cholesterol in vesicles. It appears that a high CSI is only one of the factors determining vesicle stability and cholesterol nucleation. However, bile is a complex mixture of lipids, electrolytes, mucus, proteins and other substances, all of which can play a role in promoting or inhibiting the nucleation of cholesterol from cholesterol-rich vesicles. In the other four groups, the CSI were less than unity, and vesicles were not detected. However, high cholesterol saturation of bile alone does not account for the presence of vesicles in these animals. Recently, Chijiiwa *et al.* (22) reported that in the bile of gallstone patients, a high concentration of cholesterol in biliary vesicles was associated with high CSI and a high biliary total protein concentration. Halpern *et al.* (4) discussed the possibility that factors promoting nucleation may affect biliary vesicle formation and aggregation. In the present study, we measured neither biliary protein concentration nor nucleation time. Again, the small volume of bile (20–50 µL) obtained from each hamster makes it difficult to perform such experiments.

The bile acid composition of the animals was affected by gender. There was an increased proportion of 6-hydroxylated bile acids [hyodeoxycholic acid (HDCA) and murideoxycholic acid (MDCA)] in the bile of male hamsters, compared to females. Although hamster bile contains small concentrations of ß-muricholic acid (23,24), the 7-dehydroxylation of the latter seems quantitatively inadequate to account for the presence of MDCA and [*via* bac-

TABLE 5

Reproducibility of the Distribution of Cholesterol Among Its Carriers in Hamster Bile After Feeding the Semipurified Diet Plus 1.2% Palmitic Acid Plus 0.3% Cholesterol for Two Weeks[a]

Group	Number of animals	Cholesterol (%)			Cholesterol/phospholipid ratio		
		Ves	s-Ves	Mic	Ves	s-Ves	Mic
A	8	26.9	14.0	59.1	2.70	1.91	0.32
B	9	34.2	12.3	53.5	2.42	1.48	0.32
C	8	34.9	19.0	46.1	2.63	1.01	0.24
Mean ± SD		32.0 ± 4.43	15.1 ± 3.48	52.9 ± 6.52	2.58 ± 0.15	1.42 ± 0.47	0.29 ± 0.05

[a]The data represent results from three groups of hamsters receiving identical diets. Vesicles and micelles were separated by gel filtration chromatography (Ref. 17). See Materials and Methods section for details. See Table 4 for abbreviations.

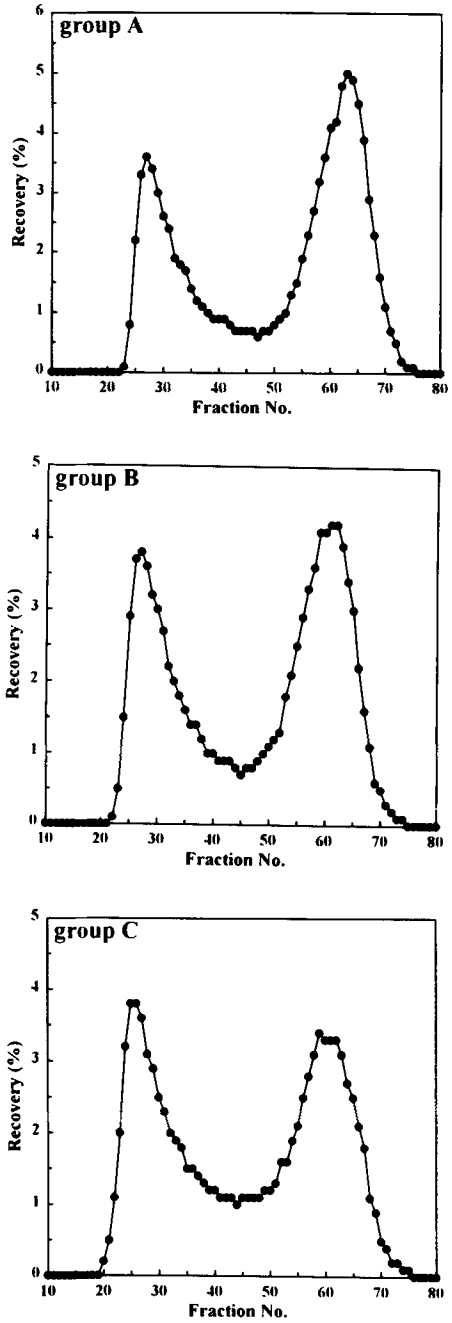

FIG. 2. Gel filtration chromatography of three samples of hamster gallbladder bile on Sepharose CL-4B-200, demonstrating reproducibility of the procedure. The elution profiles show percent recovery of [^{3}H]cholesterol (●). Hamsters were fed the semipurified diet with 0.3% cholesterol and 1.2% palmitic acid for two weeks (groups A–C). See Table 5 for quantitation. See Figure 1 for company source address.

terial action (12)] of HDCA. Further, it seems unlikely that these 6-hydroxylated bile acids arise *via* the 7-dehydroxylation of CDCA followed by 6-hydroxylation of the resulting lithocholic acid. Further studies are needed to elucidate the differences and origin of 6-hydroxy bile acids in our hamsters.

In conclusion, total biliary cholesterol concentration and the distribution of cholesterol between vesicles and micelles in bile was affected by the gender of the hamsters. Cholesterol gallstone formation (in the males) was strongly associated with the presence of vesicles in gallbladder bile. Such vesicles were absent in the females, possibly because their rates of biliary cholesterol secretion were inherently lower. These results suggest that the sex difference observed in this animal model may be related to the formation (in males) or lack of formation (in females) of vesicular cholesterol carriers. Additional studies will be needed to define the mechanism(s) underlying this difference.

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Arachidonic Acid and Eicosapentaenoic Acid Stimulate Type II Pneumocyte Surfactant Secretion

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Arachidonic acid and its leukotriene metabolites have been shown to stimulate surfactant secretion by alveolar type II cells. The present study was undertaken to determine the effects of various unsaturated fatty acids, including eicosapentaenoic acid, on surfactant secretion. Surfactant secretion was expressed as the percent of [^{3}H]choline-derived phospholipids released into culture medium by type II pneumocytes of adult rats. Consistent with the earlier findings, arachidonic acid stimulated secretion in a concentration-dependent fashion (3.5–21 μM), doubling baseline secretion at 21 μM. Eicosapentaenoic acid was found to be equally effective as arachidonic acid in stimulating secretion. A comparison with palmitic, oleic and linoleic acids revealed that highly unsaturated fatty acids stimulated secretion to the greatest extent. This was supported by a positive correlation between degree of unsaturation (i.e., 0, 1, 2, 4 and 5 double bonds) and stimulation of surfactant secretion. In the present study, exogenous leukotriene E_4 (10^{-13}–10^{-6}) did not stimulate surfactant secretion. Neither nordihydroguaiaretic acid (0.1 μM) nor indomethacin (0.1 μM) affected arachidonic acid-stimulated secretion. The stimulatory effects of arachidonic acid and eicosapentaenoic acid on surfactant secretion were related to the highly unsaturated nature of the fatty acids and were not mediated by increased levels of cyclic adenosine monophosphate or calcium.

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Surfactant is a lipoprotein complex that reduces the surface tension of water lining the lumen of the alveoli enabling respiration (1). It is synthesized, stored and secreted by the alveolar type II cells (2). Deficiency of surfactant can cause respiratory distress syndrome in premature infants and adults (3,4).

Fish oil rich in n-3 fatty acids [i.e., eicosapentaenoic (EPA) and docosahexaenoic acids] may have beneficial effects on the treatment and/or prevention of cardiovascular disease (5), cancer (6), psoriasis (7), arthritis (8) and atherogenesis (9). Despite these potential benefits, fish oil could also exert adverse effects, such as those associated with high intake of cholesterol and vitamin A and D (10), and prolonged bleeding times (5). Surfactant secretion by alveolar type II cells has been shown to be stimulated by arachidonic acid (11). One proposed mechanism underlying such stimulation involves leukotrienes derived from arachidonic acid (12). In other tissues, EPA perturbs leukotriene formation from arachidonic acid (AA) and thereby hinders the metabolic effects of AA (6–8,13,14). However, it is not known whether EPA antagonizes AA action leading to impairment of surfactant secretion. The present study was undertaken to compare the effects of EPA and AA on surfactant secretion by alveolar type II cells. The study showed that EPA is as effective as AA in stimulating surfactant secretion by alveolar type II cells.

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Abbreviations: AA, arachidonic acid; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; C-DMEM, complete Dulbecco's modified Eagle's medium; CMF–PBS, calcium-/magnesium-free phosphate-buffered saline; EPA, eicosapentaenoic acid; LTE_4, leukotriene E_4; OA, oleic acid; PA, palmitic acid.

MATERIALS AND METHODS

Cell isolation and culture. Pathogen-free, male Sprague-Dawley rats, weighing approximately 300 g, were used to isolate type II pneumocytes according to the procedure of Mason *et al.* (15) with minor modifications (16). The final step of the purification used cell differential adherence in culture. The purity of the adhering cells was 86 ± 1% (n = 4) as determined by tannic acid staining (17). The viability of the cells was 95 ± 1% (n = 10) as judged by trypan blue exclusion.

A 2-mL aliquot of cell suspension in C-DMEM (complete Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, penicillin (100 μU/mL) and streptomycin (100 μg/mL) was plated at a density of 2.0×10^6 cells/well on six-well plates and incubated for 18–20 h under an atmosphere of 5% CO_2 and 95% air saturated with water at 37°C in the presence of 0.1 mL [*methyl*-^{3}H]choline chloride (specific activity, 2.5 μCi/μmol). Lower than typical amounts of radioactivity could be used because the siliconized glassware employed minimized losses.

Immediately after the 18–20 h incubation, surfactant secretion was measured according to the procedure previously described (11,18). The adhering cells were washed three times with 2 mL of C-DMEM and then incubated at 37°C in 1.3 mL of serum-free and antibiotic-free DMEM for 30 min prior to addition of the test compounds. The cultured cells were incubated for 3 h after the test compounds had been added. In a preliminary study, a linear rate of the secretion was observed between 1 and 4 h of incubation.

The measurement of ^{3}H-labeled total lipids released into the medium is an established marker of surfactant secretion (19). Surfactant secretion was expressed as percent of the radioactivity of [^{3}H]lipid recovered in the medium over the sum of the radioactivity found in cells plus the medium multiplied by 100. Lactate dehydrogenase activity was measured (20) in the cultured cells and in the incubation media to determine if cell injury or lysis had occurred.

Preparation of test compounds. AA, EPA, linoleic acid, nordihydroguaiaretic acid and indomethacin were dissolved in ethanol, while leukotriene E_4 (LTE_4) and the

sodium salts of palmitic acid (PA) and oleic acid (OA) were dissolved in distilled water as stock solution. The solutions were diluted in serum-free and antibiotic-free DMEM prior to adding to cells. The concentration of ethanol introduced in all experiments was less than 0.05%. Ethanol at this concentration neither induced cell lysis nor stimulated surfactant secretion (unpublished data).

Measurement of cyclic adenosine monophosphate (cAMP) and intracellular calcium. The cAMP levels were measured using Amersham's cAMP[^{125}I]assay kit (Amersham Corp., Arlington Heights, IL). The cells were prepared and treated as before using two concentrations of the fatty acids, i.e., 7 and 21 μM. After the 3-h fatty acid treatment, the cells were washed with phosphate-buffered saline, and the cellular cAMP content determined.

Intracellular calcium was measured with a bioluminescent photoprotein as described elsewhere (21). The cells were preloaded with aequorin (a calcium-sensitive luminescent probe) prior to the experiment. Approximately 4–5 × 10^6 adhering cells, on a 100-mm Corning dish, were washed twice with 10 μM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF–PBS) and twice with CMF–PBS. The cells were scraped using a rubber policeman in the presence of 50 μL of 1.0 mg/mL aequorin (Friday Harbor Photoproteins, Friday Harbor, Washington). After 5 min, the cells were diluted with phenol red-free DMEM to a concentration of 1–2 × 10^6 per mL, and 0.8 mL of this cell suspension was injected into Sykes-Moore chambers (Bellco, Vineland, NJ). After a 3-h adhering time, the floating cells along with the cell debris, were washed away. The cells were then treated with the various fatty acids, and luminescence was recorded for 5 min. An established intracellular calcium mobilizer, adenosine triphosphate (ATP), was used as a positive control (22).

Culture media and chemicals. DMEM, fetal bovine serum and antibiotics were purchased from Gibco Labs (Grand Island, NY). [*Methyl*-^{3}H]choline chloride was obtained from Amersham Corporation. Ecoscint counting solvent was from National Diagnostics (Manville, NJ). LTE_4 was a gift from Merck Frosst Centre for Therapeutic Research (Pointe Claire, Dorval, Quebec, Canada). Organic solvents were reagent grade and purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and compounds were from Sigma Chemical Co. (St. Louis, MO).

Data and statistical analysis. For each cell isolation, the type II cells from two to three lungs were combined and distributed in culture plates consisting of six wells (4-cm diameter). The control and treatment groups were analyzed in triplicate for each isolation. The number of isolations for each of the different experiments are reported along with the mean and standard error, and were analyzed statistically. A one-way analysis of variance was used (Minitab Statistical Software Program, State College, PA). In a series of experiments evaluating the effects of saturated and unsaturated fatty acids on surfactant secretion, the error variance was tested by Bartlett's test for homogeneity (23), and the error variance was found to be heterogeneous. Therefore, a $\log_{10}$ transformation of the data was performed before the analysis of variance. This was followed by Fisher's least significant test (24) to determine where the difference existed.

To establish a linear relationship between the independent and dependent variables in some of the experiments, a regression analysis was performed and the correlation coefficient was obtained. Covariate analysis was used to test the difference between the slope and intercept of two regression lines (Minitab Statistical Software Program).

RESULTS

AA, EPA and surfactant secretion. The type II pneumocytes isolated and maintained in culture under the experimental conditions were responsive to known secretagogues. When treated with 10 μM ATP and 1 mM adenosine, the rates of surfactant secretion were stimulated by 178 and 82%, respectively. These results are similar to those reported by Gilfillan and Rooney (25). Under the same experimental conditions, both AA and EPA stimulated secretion in a concentration dependent fashion with a correlation coefficient of 0.73 and 0.79 for AA (four experiments) and EPA (three experiments), respectively (Fig. 1). A significant linear regression line was found for both fatty acids at a level of $P < 0.001$ for the individual experiments as well as when combined. A polynomial model did not significantly improve the fit of the data, suggesting that a linear relationship best represents the data. A saturation effect could not be observed for these fatty acids as concentrations at approximately 50 μM or greater caused cell lysis, as was indicated by cell detachment and

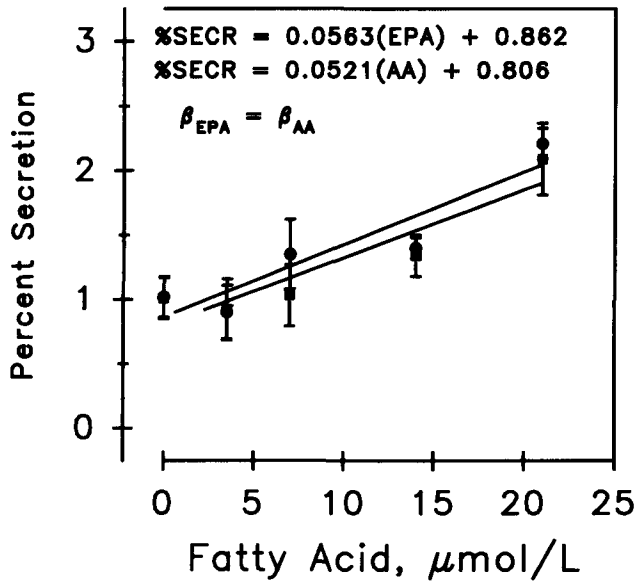

FIG. 1. A comparison of the effect of arachidonic acid (AA) and eicosapentaenoic acid (EPA) on surfactant secretion. Values are means ± SE of data from three and four cell isolations for EPA (●) and AA (■), respectively. Data from each isolation were determined in triplicate. The slope and intercept for the two regression lines are not different. Percent secretion (%SECR) is defined as the amount of [^{3}H]lipid radioactivity recovered in the media divided by the radioactivity of [^{3}H]lipid found in the cells plus the media multiplied by 100.

permeation of trypan blue into the nuclei. Lactate dehydrogenase release at the lower fatty acid concentrations used in these experiments was not different from control values (i.e., 2.6 ± 0.5% of total cellular enzyme activity, mean ± SE, n = 3), suggesting that release of surfactant was by a secretory process and did not result from cell lysis.

A further comparison of the regression lines derived from the secretory responses of AA and EPA by covariate analysis revealed that the two response curves shared the same slope and intercept (F = 0.029). This indicates that AA and EPA are equally effective in promoting surfactant secretion.

Effects of other fatty acids. In order to determine whether the stimulation of surfactant secretion is specific to highly unsaturated fatty acids, the effects of linoleic acid, OA and PA at 21 μM concentrations were also investigated (Fig. 2). Consistent with observations made in the preceding experiments, EPA and AA stimulated surfactant secretion. The stimulation by the polyunsaturated fatty acids, EPA, AA and linoleic acid, were 116, 85 and 50% above baseline, respectively. The stimulatory effect of these fatty acids was significantly greater than that caused by PA. It should be noted that the percent stimulation caused by EPA was significantly higher than that caused by linoleic acid which, in turn, was significantly higher than that elicited by PA.

Eicosanoids and surfactant secretion. AA-derived LTE_4 has previously been shown to stimulate surfactant secretion (12). The results shown in Figure 3 appeared to indicate an inhibition of surfactant secretion by LTE_4 ranging from pM to μM concentrations. However, because the slope of the regression line did not significantly differ from that for the untreated group, the data suggested that lipid secretion by the type II pneumocytes was not affected by LTE_4.

A comparison was made between groups of cells treated with AA (21 μM) alone or in combination with 0.1 μM nordihydroguaiaretic acid or 0.1 μM indomethacin. The concentration of nordihydroguaiaretic acid chosen for this experiment has been previously shown to impair AA-stimulated secretion (11). The indomethacin concentration used in this experiment has been shown to inhibit prostaglandin synthesis in other cells (26). At higher concentrations, indomethacin inhibits both prostaglandin and leukotriene synthesis (26). Therefore, we only tested indomethacin at the lower concentration. Neither the lipoxygenase nor the cyclooxygenase inhibitor altered AA-stimulated surfactant secretion at these concentrations (Fig. 4).

The roles of cAMP and calcium. When the cells were treated for 3 h with fatty acids at 7 or 21 μM concentration, there was no consistent trend of the effect of fatty acid on the respective cAMP level (Fig. 5). At the lower concentration, PA increased cellular cAMP content,

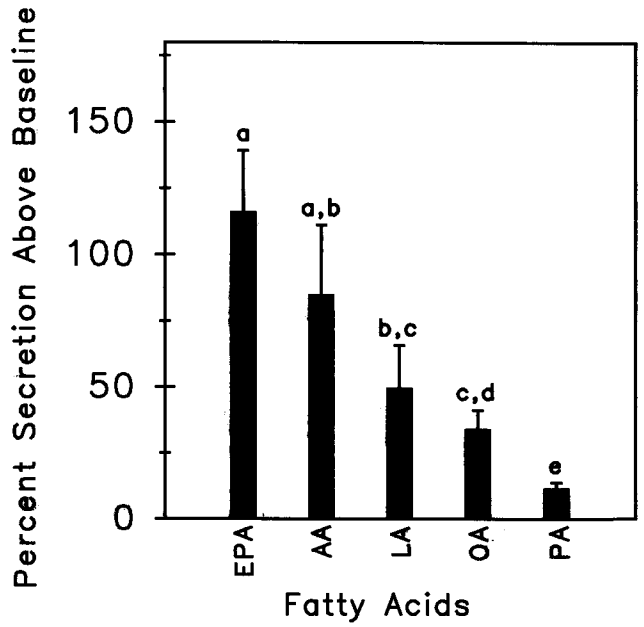

FIG. 2. The effects of different fatty acids on surfactant secretion. The concentration of fatty acids were 21 μM. Values are means ± SE for three to five cell isolations. Data from each isolation was determined in triplicate. Percent secretion above baseline was calculated by the equation: difference in percent secretion between tested and control group/percent secretion of control group × 100. The average baseline secretion was 0.93 ± 0.27%. An analysis of variance was performed with transformed data followed by a Fisher's least significant difference test (see Materials and Methods). Unshared superscript letters indicate significant differences among the responses of fatty acids to surfactant secretion. Abbreviations as in Figure 1; LA, linoleic acid; OA, oleic acid; PA, palmitic acid.

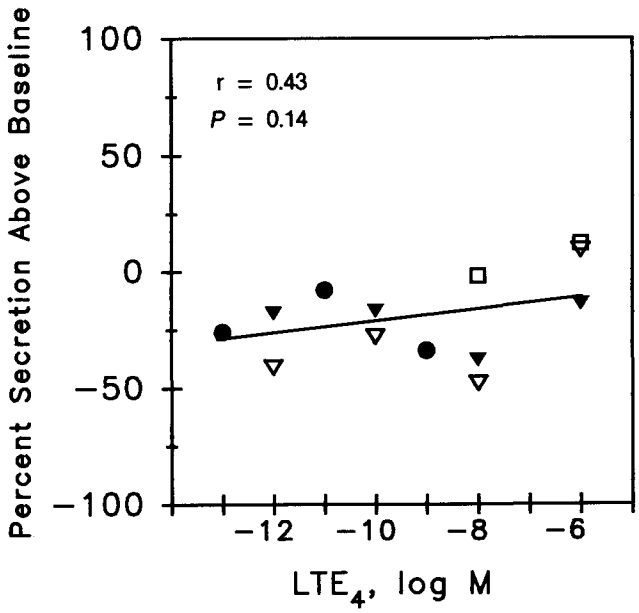

FIG. 3. The effect of leukotriene E_4 (LTE_4) on surfactant secretion. The four different symbols represent four different cell isolations. Each point represents a mean of triplicate measurements for each isolation. Percent secretion above baseline was calculated by the equation: difference in percent secretion between experimental and control group/percent secretion of control group × 100. The *P* and r represent the *P* value and correlation coefficient of the regression line, respectively.

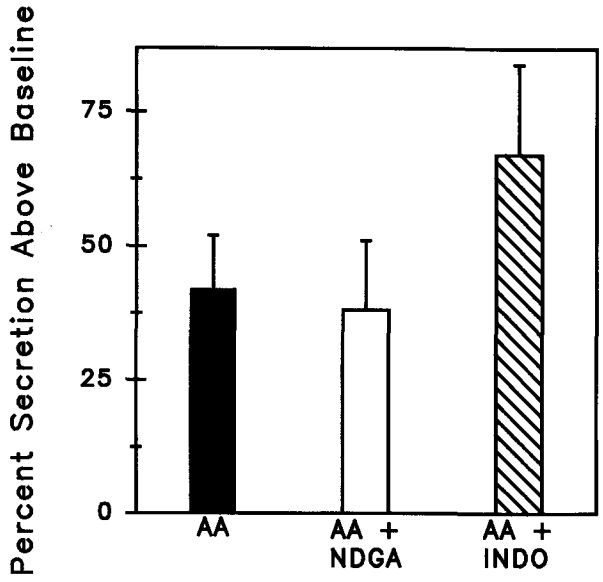

FIG. 4. The effect of a lipoxygenase inhibitor and cyclooxygenase inhibitor on arachidonic acid (AA)-stimulated surfactant secretion. The cells were incubated with 21 μM AA in the absence or presence of either 0.1 μM nordihydroguaiaretic acid (NDGA) or 0.1 μM indomethacin (INDO). Values are means ± SE for three cell isolations. Data from each isolation were determined in triplicate. Percent secretion above baseline was calculated by the equation: difference in percent secretion between experimental and control group/percent secretion of control group × 100. The average baseline secretion was 0.93 ± 0.16%.

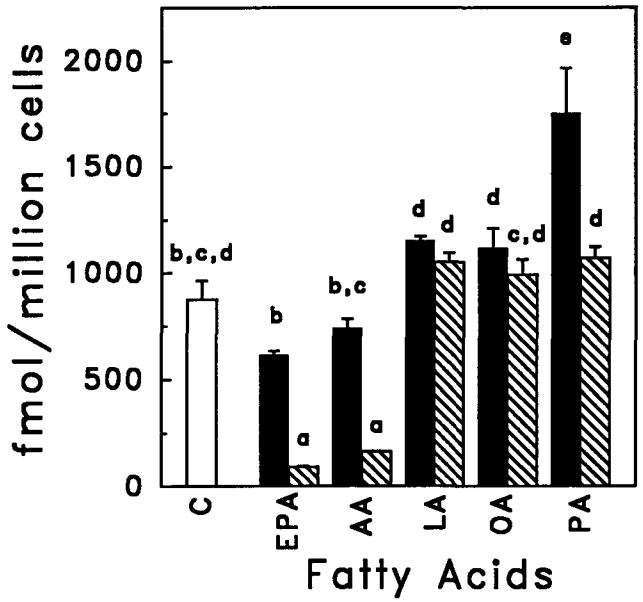

FIG. 5. The effects of different fatty acids on cyclic adenosine monophosphate (cAMP) levels. Values are means ± SE for triplicate samples from one experiment. Unshared superscript letters indicate significant difference in levels of cAMP among the cells treated with various fatty acids. Black and striped bars indicate cells treated with 7 and 21 μM fatty acid, respectively. Abbreviations as in Figure 2; C, control.

whereas the highly unsaturated fatty acids slightly decreased it. However, at the higher concentration, the highly unsaturated fatty acids (i.e., EPA and AA) markedly reduced cAMP, while no effect was noted for the other fatty acids.

The cells treated with ATP as a positive control had cytoplasmic-free calcium increased to 463 from 73 nM at baseline level. The extent of the stimulation was comparable to that reported by Dorn *et al.* (22). Despite the apparent response of the cells to ATP, the cellular-free calcium level was not altered by any of the fatty acids tested (data not shown).

DISCUSSION

Surfactant secretion is known to be regulated by a number of secretagogues, including calcium (27), β-adrenergic agonists (28) and purines (29,30). The role of AA in modulating the secretion, however, has not been fully established. Gilfillan and Rooney (11) first reported a stimulation of surfactant secretion by AA in alveolar type II cells, but such stimulatory effects were not observed by Sano *et al.* (18). Consistent with the former study, we found a significant increase in surfactant lipid secretion by AA in the cultured alveolar type II cells, and the stimulatory action of AA was found to be concentration-dependent. In view of the known antagonistic action of n-3 and n-6 fatty acid in metabolic processes (6–8,13,14), the present study was expanded to investigate the effect of EPA on surfactant secretion. Contrary to our expectations, EPA was as effective as AA in enhancing surfactant secretion in the cultured type II cells.

The mechanisms underlying stimulation of secretion by EPA and AA are not fully understood. AA-stimulated secretion has been partly attributed to lipoxygenase products derived from AA (11,12). This mechanism, however, is not supported by the data of the present study for the following reasons. First, despite a wide range of concentrations tested, exogenous LTE_4 did not alter the baseline secretion of surfactant lipid. This finding is consistent with the results of an earlier study, which had shown that leukotriene C_4 and leukotriene D_4 were ineffective in stimulating surfactant secretion (18). Second, in the present study, AA-stimulated secretion was not affected by lipoxygenase nor cyclooxygenase inhibitors. Furthermore, if leukotrienes were involved, one should have seen opposing effects of EPA and arachidonic acid since five-series leukotrienes derived from EPA are known to be less biologically active than the four-series leukotrienes generated from AA (31,32). However, because other eicosanoid inhibitors and concentrations were not thoroughly investigated, a role of leukotrienes and/or prostaglandins cannot be entirely ruled out. The reason for the discrepancy between our findings and those of Gilfillan and Rooney (11,12) in regard to the role of leukotrienes in surfactant secretion by alveolar type II cells is not clear, although they also found that indomethacin at the concentrations used in our experiments did not affect secretion (11). It should also be noted that eicosanoid-independent AA-stimulated secretion has been demonstrated in GH_3 pituitary cells (33). In these secretory cells, AA-stimulated

prolactin secretion was not reduced by leukotriene or prostaglandin inhibitors (33).

The most important finding of the present study was the differential stimulatory effect on surfactant secretion exhibited by fatty acids varying in chain length and in the number of double bonds. The level of stimulation produced was highest for highly unsaturated fatty acids (EPA and AA), lowest for the saturated PA, and intermediate for monounsaturated (oleic) and diunsaturated (linoleic) fatty acids. Thus, the stimulation of surfactant secretion is dependent on the degree of unsaturation of the fatty acids as supported by an analysis of the data which revealed a positive correlation ($r = 0.71$, $P < 0.01$) between the degree of unsaturation (e.g., 0, 1, 2, 4 and 5 double bonds) and the stimulation of secretion. This is consistent with the earlier finding by Kolesnick *et al.* (33) which showed that prolactin secretion by pituitary cells was stimulated to a greater extent by AA followed, in decreasing order, by linolenic acid, linoleic acid, OA and steric acid. However, our data do not exclude the possibility that the stimulation may also be related to carbon chain length, as there was a significant correlation ($r = 0.67$, $P < 0.01$) between chain length (e.g., 16, 18 and 20 carbons) and stimulation.

The physiological significance of fatty acid-enhanced release of surfactant is not known. Tissue fatty acid composition is susceptible to modification by dietary fat. In a recent study, we demonstrated that menhaden oil supplementation in diet increased the levels of highly unsaturated fatty acids, e.g., EPA and docosahexaenoic acid, in type II pneumocyte phospholipids (34). If polyunsaturated fatty acids enhance surfactant release relative to saturated fats, the composition of the diet may be an important factor to consider for those who have problems with surfactant production and/or release.

The precise mechanism by which highly unsaturated fatty acids stimulate surfactant secretion is unclear. Calcium and cAMP are known to enhance surfactant secretion (27,28,35), but changes in cellular levels of these two mediators are not likely to be responsible for the observed stimulation. The mechanism may rather be related to changes in membrane fluidity, which, when increased, may facilitate fusion of surfactant with the cell membrane. In fact, highly unsaturated fatty acids, such as AA, have been demonstrated to facilitate fusion of secretory vesicles into cell membranes (36). Conversely, when AA was replaced by OA, the rate of fusion was markedly reduced (36). Whether enhanced fusion of vesicles within the alveolar type II cells by highly unsaturated fatty acids promotes surfactant secretion remains to be determined.

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Evidence That Palmitic Acid Is Absorbed as *sn*-2 Monoacylglycerol from Human Milk by Breast-Fed Infants

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Milk fatty acids consist of about 20–25% palmitic acid (16:0), with about 70% of 16:0 esterified to the *sn*-2 position of the milk triacylglycerols. Hydrolysis of dietary triacylglycerols by endogenous lipases produces *sn*-2 monoacylglycerols and free fatty acids, which are absorbed, reesterified, and then secreted into plasma. Unesterified 16:0 is not well absorbed and readily forms soaps with calcium in the intestine. The positioning of 16:0 at the *sn*-2 position of milk triacylglycerols could explain the high coefficient of absorption of milk fat. However, the milk lipase, bile salt-stimulated lipase, has been suggested to complete the hydrolysis of milk fat to free fatty acids and glycerol. These studies determined whether 16:0 is absorbed from human milk as *sn*-2 monopalmitin by comparison of the plasma triacylglycerol total and *sn*-2 position fatty acid composition between breast-fed and formula-fed term gestation infants. The human milk and formula had 21.0 and 22.3% of 16:0, respectively, with 54.2 and 4.8% 16:0 in the fatty acids esterified to the 2 position. The plasma triacylglycerol total fatty acids had 26.0 ± 0.6 and 26.2 ± 0.6% of 16:0, and the *sn*-2 position fatty acids had 23.3 ± 3.3 and 7.4 ± 0.7% of 16:0 in the three-month-old exclusively breast-fed (n = 17) and formula-fed (n = 18) infants, respectively. Marked differences were found in the plasma total and the 2 position phospholipid percentage of 20:4ω6, i.e., 11.6 ± 0.3 and 6.9 ± 0.6 (total), 17.7 ± 1.4 and 9.7 ± 0.6 (*sn*-2 position) and percentage of 22:6ω3, 4.6 ± 0.3 and 2.1 ± 0.3 (total), 5.6 ± 0.6 and 2.0 ± 0.2 (*sn*-2 position) for the breast-fed and formula-fed infants, respectively. These studies provide convincing evidence that 16:0 is absorbed from human milk as *sn*-2 monoacyl-glycerol. The metabolic significance of the differences in positional distribution of fatty acids in the plasma lipids of breast-fed and formula-fed infants is not known.

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Palmitic acid (16:0) usually represents 20–25% of human milk fatty acids, and about 70% of 16:0 is esterified to the *sn*-2 position in the triacylglycerols (1–3). The reason for the preferential esterification of 16:0 to the 2 position of glycerol during triacylglycerol synthesis in the mammary gland, and any potential physiological significance of this to infant nutrition, is not yet clear. It is known that the products of gastric and pancreatic colipase-dependent lipase hydrolysis of dietary fat are free fatty acids, released from the *sn*-1,3 positions and *sn*-2 monoacylglycerols (4). The efficiency of absorption of unesterified 16:0 by infants is relatively low, only about 63% compared to over 90% for the unsaturated fatty acids oleic acid (18:1ω9) and linoleic acid (18:2ω6) (5). This is believed to be due in part to a melting point above body temperature (63°) and a tendency for rapid formation of hydrated fatty acid soaps at the pH of the intestine (4). Consistent with this, studies have been reported that showed that fat absorption is higher in infants fed fats with 16:0 esterified to the triacylglycerol *sn*-2 rather than the *sn*-1,3 positions (6–8). This supports the hypothesis that the high efficiency of human milk fat absorption may be the result of the specific positioning of milk 16:0 at the *sn*-2 position of the triacylglycerol moiety (8).

The significance of *sn*-2 monoacylglycerol absorption in infants fed fresh human milk is, however, controversial because of the hypothesized role of the milk lipase, bile salt-stimulated lipase, in milk fat digestion. This enzyme is synthesized in the mammary gland, survives the acid environment of the stomach, and, at least *in vitro*, is able to complete the hydrolysis of milk triacylglycerols to form glycerol and free fatty acids (9–13). Additional hydrolysis of the products of endogenous lipase digestion of milk fat by bile salt-stimulated lipase has been suggested to explain the high efficiency of absorption of human milk (9–13). Hydrolysis to release 16:0 from *sn*-2 monoacylglycerols formed by endogenous lipase activity, however, does not seem consistent with the specific positioning of 16:0 at the *sn*-2 position of human milk triacylglycerols, the low coefficient of absorption of unesterified 16:0, or the high coefficient of absorption of human milk fat in infants.

In contrast to human milk, 16:0 in vegetable oils and nonmilk-derived animal fats, other than lard, is predominantly esterified to the triacylglycerol *sn*-1,3 positions, and unsaturated fatty acids occupy the *sn*-2 position (2). Since endogenous lipases do not hydrolyze the *sn*-2 ester bond of dietary fats (4,9), it is reasonable to assume that infants fed formula will absorb *sn*-2 monoacylglycerols containing unsaturated fatty acids. The composition of monoacylglycerols absorbed from the intestinal lumen is important to the fatty acid distribution of circulating lipids because about 70% of the fatty acids absorbed as *sn*-2 monoacylglycerols are conserved in the original position during reesterification to form triacylglycerols in the intestinal cells (4). Several studies have shown that the rate of hydrolysis and composition of remnant particles formed by lipoprotein and hepatic lipase is influenced by the plasma triacylglycerol fatty acid composition and positional distribution (4,14,15). It is possible, therefore, that the distribution of fatty acids in milk or formula triacylglycerols could have important metabolic effects beyond that on fat absorption. As a first step in understanding the metabolic significance of the fatty acid distribution in milk fat, the present studies sought to determine if *sn*-2 monoacylglycerol containing 16:0 is absorbed from human milk. This was done by the comparing of the composition of total and *sn*-2 position fatty acids in plasma lipids between breast-fed infants and infants fed formula containing

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Abbreviation: LCAT, lecithin:cholesterol acyltransferase.

similar amounts of 16:0 to milk but predominantly esterified to the formula fat *sn*-1,3 positions.

MATERIALS AND METHODS

Subjects and methods. Infants were eligible for this study if they were full-term (>37 wk gestation at birth), appropriate weight for gestational age, and if the mother had chosen to exclusively breast-feed or feed formula for at least 3 mon. The infants were enrolled into the study by 14 d of age following written, informed parental consent prior to 14 d of age. Infants assigned to the formula group were fed a ready-to-feed liquid formula (Enfalac; Mead Johnson Nutritionals, Evansville, IN) containing amounts of 16:0 similar to those in human milk (Table 1). Body weight, length, head circumference and skin-fold thickness (subscapular, abdominal and triceps) were recorded for all infants at 14 d and at 1,2 and 3 mon of age. Breast-feeding mothers provided a mid-feed sample of breast milk at 14 d and 3 mon *postpartum*. Blood samples were obtained by venipuncture from each infant at 3 mon (90 ± 4 d) of age during an out-patient visit to the BC's Children's Hospital. Infants were not fasted, and had been fed between 1 and 3 h prior to blood sampling. The protocol and procedures involving the infants were approved by the University of British Columbia Screening Committee for Research Involving Human Subjects, and by the Research Screening Committee of the BC's Children's Hospital.

Following collection, blood samples were immediately placed on ice and transferred to the research laboratory no more than 15 min after collection. Plasma was separated by centrifugation, total lipids were extracted, and triacylglycerols, phospholipids and cholesteryl esters were separated by thin-layer chromatography (16,17). Analysis of the composition of fatty acids at the *sn*-2 position of the plasma phospholipids and triacylglycerols, and of the formula and milk fat was based on the methods published by Kuksis (18) and Christie (19), as described elsewhere (17). The enzymatic hydrolysis utilized pig pancreatic lipase (EC 3.1.1.3, type II) for the milk and formula fat and plasma triacylglycerols, and phospholipase A_2 (EC 3.1.1.4) (Sigma Chemical Co., St. Louis, MO) for plasma phospholipids. After hydrolysis, 17:0 monoacylglycerol was added as an internal standard, the sample was extracted, and the monoacylglycerols separated by thin-layer chromatography. Monoacylglycerol recovery and the composition of the fatty acids in the *sn*-2 position of the milk and formula diet, and in the plasma lipid fractions was determined by fatty acid methyl ester analysis on the monoacylglycerol fraction. The fatty acid compositions of the milk and formula fat were analyzed by the method of Lepage and Roy (20) without solvent extraction. Fatty acid methyl esters were analyzed using a Varian 3400 gas–liquid chromatograph equipped with an RTx 2330 capillary column, 30 m x 25 mm i.d. (Restek Corporation, Bellefonte, PA) and a Varian Star data system (Varian Canada Inc., Georgetown, Ontario, Canada) as described elsewhere (17). The flow rate of the carrier gas (helium) was 1 mL/min; the injector and flame-ionization detector were maintained at 240 and 260°C, respectively. The samples were injected with the column oven at 80°C. The temperature of the oven was programmed to increase to 170°C at 10°C/min after 2 min, then increase to 195°C at 20°C/min after 25 min, and to increase to 245°C at 20°C/min after 18 min. The fatty acid methyl esters were identified by comparison of their retention times with those of authentic standards.

Statistical analysis. Comparisons of results to determine the effect of breast-feeding vs. formula-feeding on the fatty acid compositions of the infants' plasma lipids were conducted using Student's *t*-test with a $P < 0.05$ as statistically significant. All calculations were performed using the GLM procedure in the Number Cruncher Statistical System, version 5.01 (Kaysville, UT).

TABLE 1

Composition of Human Milk and Infant Formula Fat Total and *sn*-2 Position Fatty Acids (wt%)[a]

Fatty acid (wt%)	Human milk		Formula	
	Total	*sn*-2 Position	Total	*sn*-2 Position
12:0	4.1 ± 0.4	2.5 ± 0.4	8.9	4.5
14:0	5.5 ± 0.4	6.2 ± 0.8	4.7	1.0
16:0	21.0 ± 0.5	54.2 ± 1.5	22.3	4.8
16:1	3.1 ± 0.2	3.5 ± 0.3	0.2	0.2
18:0	7.1 ± 0.3	2.9 ± 0.4	5.1	1.3
18:1	40.2 ± 0.7	17.1 ± 0.8	37.1	58.8
18:2ω6	13.4 ± 0.8	8.1 ± 0.7	17.9	27.1
18:3ω3	1.5 ± 0.1	0.9 ± 0.1	2.1	1.8
20:2ω6	0.4 ± 0.0	0.1 ± 0.0	nd	nd
20:3ω6	0.4 ± 0.0	0.2 ± 0.0	nd	nd
20:4ω6	0.5 ± 0.0	0.7 ± 0.1	nd	nd
20:5ω3	0.1 ± 0.0	0.1 ± 0.0	nd	nd
22:4ω6	0.1 ± 0.0	0.2 ± 0.0	nd	nd
22:5ω3	0.2 ± 0.0	0.3 ± 0.0	nd	nd
22:6ω3	0.2 ± 0.0	0.4 ± 0.0	nd	nd

[a]Values given are percentage of total fatty acids and for human milk represent means ± SEM (range) of mid-feed milk collected when the infants were 3 months old. Values for formula are the average of three separate analyses; <0.1 indicates value >0.00 and <0.10%; nd, not detected.

RESULTS

Formula and milk triacylglycerol composition and fatty acid distribution. The fatty acid composition of the human milk samples was similar to that reported in other recent studies (2) with mean levels of 21.0% 16:0, 13.3% 18:2ω6, 0.5% 20:4ω6 and 0.2% 22:6ω3 (Table 1). The fatty acids at the *sn*-2 position of the milk fat had 54.2% 16:0, indicating that over 80% of the milk total 16:0 was esterified to the *sn*-2 position. This result is consistent with the value of 51.2% 16:0 in fatty acids at the *sn*-2 position of mature human milk triacylglycerols reported by Martin *et al.* (3). In contrast to 16:0, only about 14% of the total 18:1 and 20% of the total 18:2ω6 in the milk fat was esterified to the *sn*-2 position (Table 1). The longer chain ω6 and ω3 fatty acids, 20:4ω6 and 22:6ω3, were approximately equally distributed between the *sn*-2 and the *sn*-1/-3 positions, with about 46%

of the 20:4ω6 and 66% of the 22:6ω3 being recovered in the *sn*-2 monoacylglycerols (Table 1). Martin *et al.* (3) used chemical degradation to show that about 49% of the 20:4ω6 and 66% of the 22:6ω3 was in the *sn*-2 position, and about 45% of the 20:4ω6 and 33% of the 22:6ω3 was esterified to the *sn*-3 position of human milk triacylglycerols. The infant formula contained an amount of 16:0 (22.3%) similar to that in human milk. The formula, however, contained predominantly 18:1 and 18:2ω6, and had only 4.8% of 16:0 in the fatty acids esterified to the 2 position of the formula fat.

Total and sn-2 *fatty acid composition of plasma triacylglycerols from breast-fed infants and infants fed formula.* These studies provide unambiguous evidence that 16:0 esterified to the *sn*-2 position of human milk is absorbed intact by human infants and re-esterified to triacylglycerols for secretion into plasma (Table 2). The triacylglycerol total fatty acids of the breast-fed infants had significantly higher levels of 14:0, 16:1, 18:0, 20:4ω6, 20:5ω3 and 22:5ω6 and lower levels of 18:2ω6 than the formula-fed infants. The significantly higher percentage of 16:0 in the triacylglycerol *sn*-2 position fatty acids of the three-month-old breast-fed compared to formula-fed infants (23.3 ± 3.3 and 7.4 ± 0.7% 16:0, respectively) was accompanied by significantly lower levels of 18:1 and 18:2ω6. The formula-fed infants also had significantly lower levels of 16:1, 20:3ω6, 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3 in their plasma triacylglycerol *sn*-2 position fatty acids than did the breast-fed infants.

Total and sn-2 *fatty acid composition of plasma phospholipids from breast-fed infants and infants fed formula.* The plasma phospholipid total fatty acids of the breast-fed infants had significantly lower levels of 16:0 and 18:2ω6, but higher levels of 18:0, 20:2ω6, 20:3ω6, 20:4ω6, 20:5ω3, 22:4ω6, 22:5ω3 and 22:6ω3 than those of infants who were fed formula (Table 3). Similarly, the phospholipid *sn*-2 position fatty acids of the breast-fed infants had significantly lower levels of 18:2ω6 and 18:3ω3 and higher 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3 than those of the formula-fed infants.

Fatty acid composition of plasma cholesteryl esters from breast-fed infants and infants fed formula. The major difference in the plasma cholesteryl ester fatty acid composition due to feeding formula rather than breast feeding was in the percentage of 20:4ω6 and 22:6ω3. Levels of 20:4ω6 and 22:6ω3 in the plasma cholesteryl esters of the infants who were fed formula were approximately one-third of the levels in the infants who were breast-fed. The cholesteryl ester percentages of 14:0, 16:0, 16:1 and 20:5ω3 were also significantly lower, whereas the percentage of 18:2ω6 and 18:3ω3 were significantly higher in the formula-fed than breast-fed infants.

TABLE 2

Composition of Plasma Triacylglycerol Total and *sn*-2 Position Fatty Acids in Three-Month-Old Breast-Fed and Formula-Fed Gestation Infants[a]

Fatty acid (wt%)	Triacylglycerol total		*sn*-2 Position	
	Breast-fed	Formula-fed	Breast-fed	Formula-fed
12:0	0.5 ± 0.1	1.0 ± 0.2	0.2 ± 0.2	0.5 ± 0.1
14:0	2.9 ± 0.3	2.2 ± 0.2[b]	1.8 ± 0.8	0.7 ± 0.2
16:0	26.0 ± 0.6	26.2 ± 0.6	23.3 ± 3.3	7.4 ± 0.7[c]
16:1	2.7 ± 0.1	1.2 ± 0.0[b]	2.6 ± 0.3	1.3 ± 0.2[c]
18:0	6.6 ± 0.3	5.2 ± 0.2[b]	4.4 ± 0.5	6.0 ± 1.0
18:1	44.0 ± 0.6	42.7 ± 0.6	41.8 ± 2.5	53.3 ± 1.6[c]
18:2ω6	12.8 ± 0.8	17.7 ± 0.4[b]	19.5 ± 1.7	27.4 ± 0.6[c]
18:3ω3	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
20:2ω6	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
20:3ω6	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0[c]
20:4ω6	0.8 ± 0.1	0.3 ± 0.0[b]	2.0 ± 0.3	0.5 ± 0.1[c]
20:5ω3	0.1 ± 0.0	0.0 ± 0.0[b]	0.1 ± 0.0	0.0 ± 0.0[c]
22:4ω6	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
22:5ω6	0.1 ± 0.0	0.0 ± 0.0[b]	0.0 ± 0.0	0.0 ± 0.0
22:5ω3	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.2	0.1 ± 0.0[c]
22:6ω3	0.3 ± 0.0	0.2 ± 0.0	0.9 ± 0.1	0.1 ± 0.0[c]

[a]Values given are means ± SEM for seventeen breast-fed and eighteen formula-fed infants.
[b,c]Value for total or *sn*-2 fatty acids, respectively, significantly different from respective values for breast-fed infants; $P < 0.05$.

TABLE 3

Composition of Plasma Phospholipid Total and *sn*-2 Position Fatty Acids (wt%) in Infants Fed Human Milk or Formula[a]

Fatty acid (wt%)	Phospholipid total		*sn*-2 Position	
	Breast-fed	Formula-fed	Breast-fed	Formula-fed
12:0	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.2	0.3 ± 0.1
14:0	0.4 ± 0.1	0.4 ± 0.0	1.0 ± 0.2	0.8 ± 0.1
16:0	26.1 ± 0.6	28.4 ± 0.6[b]	13.4 ± 1.5	12.4 ± 1.0
16:1	0.5 ± 0.1	0.4 ± 0.0	0.8 ± 0.1	0.7 ± 0.1
18:0	18.4 ± 0.4	17.7 ± 0.2[b]	10.8 ± 1.3	9.5 ± 0.7
18:1	12.5 ± 0.3	12.6 ± 0.2	14.4 ± 0.6	15.3 ± 0.3
18:2ω6	19.8 ± 0.7	26.4 ± 1.0	25.0 ± 1.4	40.2 ± 1.6[c]
18:3ω3	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0[c]
20:2ω6	0.4 ± 0.0	0.3 ± 0.0[b]	0.5 ± 0.0	0.4 ± 0.0
20:3ω6	2.8 ± 0.1	2.3 ± 0.1[b]	4.5 ± 0.3	3.6 ± 0.1[c]
20:4ω6	11.6 ± 0.3	6.9 ± 0.6[b]	17.7 ± 1.4	9.7 ± 0.6[c]
20:5ω3	0.4 ± 0.0	0.2 ± 0.0[b]	0.6 ± 0.1	0.2 ± 0.1[c]
22:4ω6	0.4 ± 0.0	0.3 ± 0.0[b]	0.8 ± 0.0	0.9 ± 0.1
22:5ω6	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.1
22:5ω3	1.0 ± 0.0	0.5 ± 0.1[b]	1.5 ± 0.1	0.6 ± 0.0[c]
22:6ω3	4.6 ± 0.3	2.1 ± 0.3[b]	5.6 ± 0.6	2.0 ± 0.2[c]

[a]Values given are means ± SEM for seventeen breast-fed and eighteen formula-fed infants.
[b,c]Value for total or *sn*-2 fatty acids, respectively, significantly different from respective infants; $P < 0.05$.

DISCUSSION

The results of these studies provide convincing evidence that 16:0 esterified to the *sn*-2 position of human milk is conserved throughout digestion and absorption, and the conversion to triacylglycerols in the enterocyte, and then secreted into plasma as lipoprotein triacylglycerols. *In vitro* studies have shown that the milk lipase, bile salt-stimulated lipase, can complete the digestion of *sn*-2 monoacylglycerols formed by gastric acid colipase-dependent lipase hydrolysis of triacylglycerols to give unesterified fatty acids and glycerol (9,13). Information

on the extent of hydrolysis of *sn*-2 monoacylglycerols with 16:0 *in vivo* in young infants has not been published. Strong circumstantial evidence that bile salt-stimulated lipase does not quantitatively hydrolyze 16:0 from the *sn*-2 position of human milk fat is provided by the results of these studies that show approximately 26% of 16:0 in fatty acids esterified to the plasma triacylglycerol *sn*-2 position of breast-fed infants compared to only about 7.4% 16:0 in infants fed a formula containing comparable amounts of 16:0 to the milk, but predominantly esterified to the glycerol *sn*-1,3 positions. These results are evidence in support of the hypothesis (8) that the preferential esterification of 16:0 to the *sn*-2 position of human milk triacylglycerols is one of the reasons for the high coefficient of absorption of human milk fat. Although the studies reported here indicate that bile salt-stimulated lipase does not quantitatively complete the hydrolysis of milk triacylglycerols to free fatty acids and glycerol in infants *in vivo*, other studies have suggested a possible role in the hydrolysis of milk long-chain ω6 and ω3 fatty acids. The positioning of a substantial proportion of the 20:4ω6 and 22:6ω3 at the *sn*-3 position in milk triacylglycerols (3) is believed to render these fatty acids relatively resistant to hydrolysis by pancreatic colipase-dependent lipase (21,22). Whether or not bile salt-stimulated lipase is important in the digestion of long-chain ω6 and ω3 fatty acids from human milk has still to be fully clarified.

The plasma phospholipid levels of 20:4ω6 and 22:6ω3 are known to be lower in infants fed formula than in infants fed human milk (23,24). The analyses reported here show that this difference involves substantially lower 20:4ω6 and 22:6ω3 levels, but much higher 18:2ω6 levels in the fatty acids esterified to the *sn*-2 position of plasma phospholipids in infants fed formula rather than breast-fed (mean ± SEM 18:2ω6, 40.2 ± 1.6 and 25.0 ± 1.4%, 20:4ω6, 9.7 ± 0.6 and 17.7 ± 1.4%; 22:6ω3, 2.0 ± 0.2 and 5.6 ± 0.6%; for the formula and breast-fed infants, respectively). The higher levels of 18:2ω6 at the *sn*-2 position of plasma phospholipids in the formula-fed compared to breast-fed infants seems to reflect the higher amount of 18:2ω6 at the 2 position of the formula compared to the human milk fat (27.1 and 8.1% 18:2ω6 for the formula and human milk, respectively), and not the small difference in 18:2ω6 between the formula and milk total fatty acids (17.7 and 12.8% 18:2ω6, respectively). Although recent studies have shown that phospholipid synthesis in intestinal cells may proceed *via* the monoacylglycerol pathway (25), it is not clear to what extent this occurs *in vivo* in young infants. It may also be of relevance that recent studies have shown that the incorporation of 20:4ω6 into rat lymph phospholipids was higher when 20:4ω6 was given with 18:1 and monopalmitin than when given with 18:2ω6 and monoolein (26). This may suggest that absorption of monopalmitin following hydrolysis of human milk fat could facilitate incorporation of 20:4ω6 into intestinal lipoprotein phospholipids of breast-fed infants. Future studies on the fatty acid composition and structure of intestinal chylomicron and very low density lipoprotein would provide more specific information on this.

In recent studies, higher levels of 16:0 were found in plasma cholesteryl esters of piglets fed milk or formula containing 16:0 esterified to the 2 position of the fat than in piglets fed formula with 16:0 predominantly esterified to the triacylglycerol 1,3 positions (17). A similar, but smaller difference was found in the present studies with infants; that is, the percentage of 16:0 was about 15% higher in the cholesteryl esters of the breast-fed than of the formula-fed infants. Neither the origin nor metabolic significance of the relatively high amounts of cholesteryl palmitate in plasma seems to be known. Recent studies have suggested that lecithin:cholesterol acyltransferase (LCAT) from human plasma preferentially uses the *sn*-1 acyl group from phosphatidylcholines when 20:4ω6 or 22:6ω3 is esterified to the *sn*-2 position (26). Since molecular species of phosphatidylcholines containing 20:4ω6 and 22:6ω3 in the *sn*-2 position have 16:0 and 18:0 in the *sn*-1 position, this could explain the origin of 16:0 in plasma cholesteryl esters. The higher proportions of 20:4ω6 and 22:6ω3 in the plasma cholesteryl esters of infants who are breast-fed rather than fed formula (Table 4), however, suggests LCAT is able to use these fatty acids as substrates and form cholesteryl esters. Whether or not cholesteryl esters are quantitatively important in the transfer of fatty acids to tissues, possibly *via* low density lipoprotein receptors or *via* high density lipoprotein transfer, and the significance of the fatty acid composition to cholesteryl ester metabolism is not known.

The studies reported here provide *in vivo* data to show that the distribution of saturated and unsaturated fatty acids in human milk and infant formula is a determinant of the fatty acid distribution of infant plasma triacylglycerols and phospholipids. The high amount of 16:0 in the *sn*-2 position of plasma triacylglycerols in breast-fed infants shows that 16:0 is absorbed as *sn*-2 mono-

TABLE 4

Composition of Plasma Cholesteryl Ester Fatty Acids (wt%) in Infants Fed Human Milk or Formula[a]

Fatty acid (wt%)	Breast-fed	Formula-fed
12:0	0.2 ± 0.0	0.2 ± 0.0
14:0	1.1 ± 0.0	0.8 ± 0.0[b]
16:0	17.2 ± 0.5	15.0 ± 0.6[b]
16:1	2.6 ± 0.1	1.9 ± 0.1[b]
18:0	3.1 ± 0.3	3.2 ± 0.2
18:1	25.4 ± 0.6	27.1 ± 0.7
18:2ω6	40.1 ± 0.7	45.8 ± 0.8[b]
18:3ω3	0.4 ± 0.0	0.5 ± 0.0[b]
20:2ω6	0.1 ± 0.0	0.1 ± 0.0
20:3ω6	0.7 ± 0.0	0.5 ± 0.1
20:4ω6	6.3 ± 0.3	2.3 ± 0.2[b]
20:5ω3	0.2 ± 0.0	0.1 ± 0.0[b]
22:4ω6	0.3 ± 0.1	0.5 ± 0.1
22:5ω6	0.0 ± 0.0	0.1 ± 0.0
22:5ω3	0.1 ± 0.0	0.1 ± 0.0
22:6ω3	0.6 ± 0.1	0.2 ± 0.0[b]

[a]Values given are means ± SEM for seventeen breast-fed and eighteen formula-fed infants.
[b]Values significantly different from respective values for breast-fed infants.

palmitin by infants fed human milk. In contrast, it is probable that 16:0 is absorbed predominantly as an unesterified fatty acid from conventional infant formula. It is likely that analyses of isolated chylomicrons will show higher amounts of 16:0 in the triacylglycerol *sn*-2 position than were found in plasma total triacylglycerols. Lipoprotein lipase is stereospecific and is known to hydrolyze triacylglycerols with saturated fatty acids at the *sn*-2 position more slowly than triacylglycerols with unsaturated fatty acids at the *sn*-2 position (4,14,15). Whether or not hydrolysis of plasma triacylglycerols proceeds at similar rates in infants fed formula and infants fed human milk is not known. Future work, possibly involving analysis of the complete positional distribution of fatty acids in triacylglycerols and phospholipids in separated lipoproteins may help elucidate the implications of human milk fat structure to fatty acid metabolism and tissue delivery in the infant.

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In vitro Activation of Mouse Skin Protein Kinase C by Fatty Acids and Their Hydroxylated Metabolites

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To understand how dietary fatty acids differentially modulate mouse skin tumorigenesis, the ability of specific fatty acids and their derivatives to activate murine epidermal protein kinase C (PKC) *in vitro* was investigated. Total PKC from untreated female SSIN mouse skin was partially purified and incubated with specific fatty acids at concentrations up to 300 μM in the presence of Ca^{2+} and phosphatidylserine. The *cis*-unsaturated fatty acids tested, ranging from 16:1 to 22:6, stimulated PKC activity in a similar dose-dependent manner with an approximate threefold maximum increase over control. Neither the number of *cis*-double bonds nor the chainlength of these fatty acids affected their relative ability to activate PKC. *trans*-Fatty acids, with the exception of linoelaidic acid (*t,t*-18:2n-6), exhibited about half of the potency of their corresponding *cis*-isomers in stimulating PKC at the plateau concentration (200 μM) or lower. Substitutions close to the double bond on *cis*-fatty acids abolished their ability to activate PKC. The hydroxylated metabolites of arachidonic acid (20:4n-6) and linoleic acid (*c,c*-18:2n-6), i.e., the hydroxyeicosatetraenoic acids (HETE) and hydroxyoctadecadienoic acids (HODE), also activated mouse skin PKC *in vitro*, but only about half as effectively as did the respective parent fatty acids. The results suggest that both hydroxyl substitution and *trans*-configuration of HETE and HODE are responsible for their reduced ability to activate PKC. Overall the data suggests that the reduced skin tumor yield observed in mice fed diets high in *c,c*-18:2n-6 is not likely to be due to differences in the ability of *c,c*-18:2n-6 or 20:4n-6, or their metabolites, to activate PKC.

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Protein kinase C (PKC) is a crucial enzyme in cellular signal transduction, and it is also a target receptor protein for phorbol ester (1). *In vitro* studies have shown that PKC phosphorylates a broad range of endogenous proteins containing seryl and threonyl residues (1,2). Numerous physiological functions of PKC have been determined, such as its involvement in cell secretion, exocytosis, smooth muscle contraction, gene expression and cell proliferation (3). Ten isozymes of PKC have been identified in mammalian tissues thus far; the characteristics and tissue specificity of several PKC isozymes have been reviewed by Nishizuka (4). PKC isozymes α, β, γ, δ, ε and ζ have been identified in mouse epidermal cells by different groups (5–9). PKC was first described as a Ca^{2+}- and phospholipid-dependent enzyme that could be activated by diacylglycerol (1). Various studies have shown that unsaturated fatty acid and some of their oxygenated metabolites can activate PKC isozymes from rat brain (10,11) or human placental cytosol (12) *in vitro* and in intact human platelets (13). The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which has a diacylglycerol-like structure, can increase the affinity of PKC for Ca^{2+} and activates PKC (1).

Arachidonic acid (20:4n-6) and other fatty acids can be released from phospholipids by phospholipase A_2 in many tissues and may subsequently activate PKC (1,4). Numerous studies have also shown that the composition of membrane phospholipids can be altered by modifying dietary fat composition (14–17). As shown previously, linoleic acid can compete with arachidonic acid for incorporation into epidermal cell membranes in mice fed a diet rich in corn oil (18). This, in turn, would alter the composition of the free fatty acids liberated from phospholipids by phospholipases. Fan *et al.* (13) reported that arachidonic acid and linoleic acid exhibited a similar potential to activate rat brain PKC *in vitro*; however, arachidonic acid showed a much greater ability to stimulate the phosphorylation of a 43-kDa protein than did linoleic acid in intact human platelets.

Both 20:4n-6 and *c,c*-18:2n-6 are substrates of lipoxygenase and can be metabolized to hydroxy metabolites (19). In a previous study, we have demonstrated that approximately 20% of 20:4n-6 was metabolized by lipoxygenase in mouse epidermal cells, which resulted in the *in vitro* formation of four major monohydroxy metabolites (5-,8-,12- and 15-hydroxyeicosatetraenoic acid, HETE) (20). We also tentatively identified 9- and 13-hydroxyoctadecadienoic acid (HODE) as the major metabolites when [1-^{14}C]*c,c*-18:2n-6 was incubated with mouse epidermal cells. Therefore, in addition to 20:4n-6 and *c,c*-18:2n-6, we studied the ability of the hydroxylated metabolites of 20:4n-6 and *c,c*-18:2n-6 (HETE and HODE) to activate PKC in mouse skin.

Previous work in our laboratory has demonstrated that diets high in *c,c*-18:2n-6 caused changes in the fatty acid composition of epidermal phospholipids such that *c,c*-18:2n-6 levels increased while 20:4n-6 levels decreased. The high *c,c*-18:2n-6 diets also inhibited TPA-induced skin tumor promotion, when compared to low *c,c*-18:2n-6 diets (20). While the direct activation of PKC by TPA is believed to be responsible for most, if not all, the effects of TPA, including promotion (1), other nonphorbol ester promoters, such as chrysarobin, do not directly activate PKC but do cause loss of PKC activity (21). This apparent down-regu-

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Abbreviations: ATP, adenosine 5′-triphosphate; DEAE-cellulose, diethyl-aminoethyl cellulose; 5,6-DHT, 5,6-dihydroxyeicosatrienoic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 12,13-EODE, 12(13)-epoxyoctadienoic acid; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HpODE, hydroperoxyoctadecadienoic acid; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

lation of PKC is likely to be due to the activation of PKC by ligands produced in response to chrysarobin (22). It was therefore the goal of this study to compare the relative ability of different fatty acids to activate epidermal PKC and to determine whether the activation observed correlated with the known effects of these dietary fatty acids on carcinogenesis.

MATERIALS AND METHODS

Materials. 5(*S*)-, 8(*S*)-, 12(*S*)- and 15(*S*)-HETE, 9(*S*)- or 13(*S*)-HODE, 5,6-dihydroxyeicosatrienoic acid (5,6-DHT) and 12(13)-epoxyoctadecadienoic acid (12,13-EODE) were purchased from Cayman Chemical (Ann Arbor, MI). All fatty acids tested (see Table 1), histone (Type III-S), adenosine 5′-triphosphate (ATP), phosphatidylserine (PS), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), diethylaminoethyl (DEAE) cellulose, and the protease inhibitors aprotinin, leupeptin and pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO). Whatman phosphocellulose paper, grade p81, was purchased from Fisher Scientific (Houston, TX). [γ-^{32}P]ATP (specific activity, 25 Ci/mmol; concentration 10 mCi/mL) was purchased from ICN Biomedicals, Inc. (Irvine, CA).

PKC preparation. Untreated female SSIN (SENCAR) mice (from The University of Texas M.D. Anderson Cancer Center, Science Park Veterinary Division breeding colony, Smithville, TX), each 6–12 weeks old and weighing 25–40 g were used in all experiments. Mice were shaved and then killed by cervical dislocation. The dorsal skin was removed immediately and placed on ice. Epidermal cells were scraped and placed in homogenization buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 μg/mL aprotinin, 50 μg/mL leupeptin, 10 μg/mL pepstatin and 0.5% Triton X-100). Skin scrapings were homogenized and centrifuged at 540,000 × *g* (100,000 rpm) for 10 min at 4°C using a Beckman TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA). The protein concentration of the supernatant was determined by the Coomassie blue reaction (Bio-Rad, Richmond, CA). Protein (2 mg) was loaded onto a 1-mL DEAE-cellulose column, partially purified PKC was eluted with 0.12 M NaCl.

PKC activity assay. Total PKC activity was determined as described by Ashendel *et al.* (23) and as modified by Fischer *et al.* (24). In brief, PKC activity was measured by comparing the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to histone (Type III-S) in the presence (plus) and absence (minus) of Ca^{2+} (0.5 mM) and PS (20 μg/mL). Ten μL of ethanol, or various concentrations of HETE and HODE or fatty acids in 10 μL of ethanol, were added into tubes containing 75 μL of assay mixture. Assay mixtures consisted of 20 mM Tris, pH 7.6, 10 mM $MgCl_2$, 375 μg/mL histone, and 20 μM ATP along with 20 μCi/mL [γ-^{32}P]ATP. For each assay, 25 μL of PKC eluate (elution buffer for background samples) was added into each tube at 15-s intervals and incubated at 30°C for 10 min, after which 25 μL of reaction mixture was spotted on grade p81 phosphocellulose paper (1 square inch) and then immersed into 75 mM phosphoric acid solution (10 mL per sample). After three subsequent washes with distilled water, the radioactivity of each sample was counted in 5 mL of scintillation cocktail using a Beckman LS-1801 Liquid Scintillation Counter. The specific activity of PKC was calculated as pmol phosphate incorporated/min/mg protein in the PKC eluate.

To minimize variations in the actual PKC activities between different experiments, the PKC activity of each compound was normalized using the activity induced by arachidonic acid at 300 μM as 100% activity. Data were standardized as percentage of PKC activity relative to that observed in the presence of 300 μM arachidonic acid.

Statistical analysis. Results are expressed as means ± standard errors (n = 9); data were analyzed by Fisher PLSD analysis performed at a 95% confidence level.

TABLE 1

Nomenclature of Fatty Acids

Common name of fatty acid	Shorthand	*cis/trans*[a]
Stearic acid	18:0	
Palmitoleic acid	*c*-16:1n-7	*Z*
Palmitelaidic acid	*t*-16:1n-7	*E*
cis-Vaccenic acid	*c*-18:1n-7	*Z*
trans-Vaccenic acid	*t*-18:1n-7	*E*
Oleic acid	*c*-18:1n-9	*Z*
Elaidic acid	*t*-18:1n-9	*E*
Linoleic acid	*c,c*-18:2n-6	*Z,Z*
Linoelaidic acid	*t,t*-18:2n-6	*E,E*
Linolenic acid	18:3n-3	*all Z*
γ-Linolenic acid	18:3n-6	*all Z*
Methyl γ-linolenate	Me-18:3n-6	*all Z*
Arachidonic acid	20:4n-6	*all Z*
Eicosapentaenoic acid	20:5n-3	*all Z*
Docosatrienoic acid	22:3n-3	*all Z*
Adrenic acid	22:4n-6	*all Z*
Docosahexaenoic acid	22:6n-3	*all Z*

[a]*Z*, double bond with a *cis*-configuration; *E*, double bond with a *trans*-configuration.

RESULTS

Basal PKC activity in the presence of Ca^{2+} (0.5 mM) and PS (20 μg/mL), without any addition of fatty acid, was approximately 30 pmol/min/mg protein (Fig. 1). The maximum PKC activity observed in the presence of 20:4n-6 at 200 and 300 μM was about 100 pmol/min/mg protein (Fig. 1). Stearic acid (18:0) only activated PKC minimally in that the maximum PKC stimulation caused by 18:0 was less than 40% of that caused by 20:4n-6 at 300 μM (Fig. 1).

Fatty acids differing in the number of *cis*-double bonds, namely, oleic acid (*c*-18:1n-9), *c,c*-18:2n-6, γ-linoleic acid (18:3n-6), 20:4n-6, eicosapentaenoic acid (20:5n-3) or docosahexaenoic acid (22:6n-3), activated PKC in a similar dose-response manner (Fig. 1). PKC activity reach a plateau at a concentration of 200 μM for

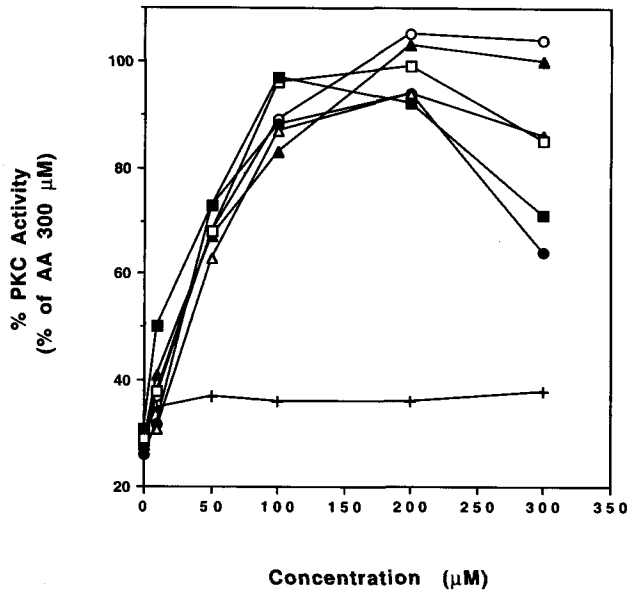

FIG. 1. A dose-response comparison of protein kinase C (PKC) activity induced by a saturated fatty acid, 18:0 (cross) or unsaturated fatty acids with different numbers of double bonds: *c*-18:1n-9 (open circles), 18:2n-6 (closed circles), 18:3n-6 (open triangles), 20:4n-6 (closed triangles), 20:5n-3 (open squares) or 22:6n-3 (closed squares). An internal standard, arachidonic acid (AA) (20:4n-6) 300 μM, was included in each assay. The PKC activity of each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 μM. Three separate experiments for each fatty acid were performed, with triplicates for each concentration.

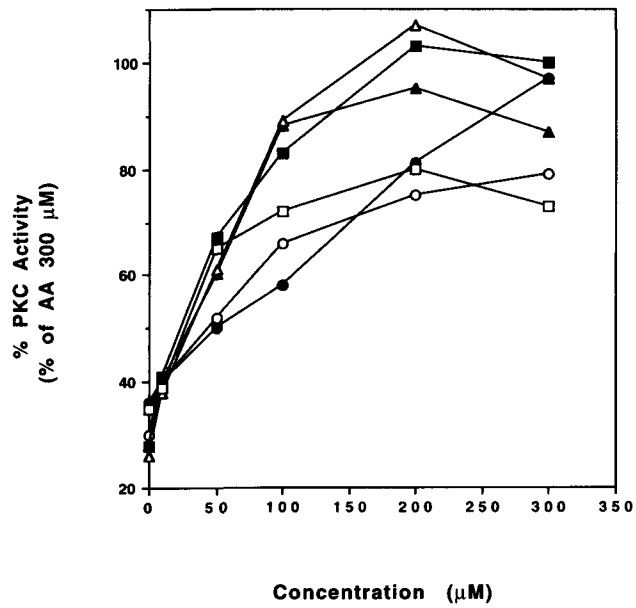

FIG. 2. Effect of chain length on the potency of *cis*-fatty acids to activate PKC. Three pairs of *cis*-fatty acids with the same number of double bonds but different chainlength were tested: *c*-16:1n-7 (closed triangles), *c*-18:1n-7 (open triangles); 18:3n-3 (closed circles), 22:3n-3 (open circles); 20:4n-6 (closed squares), 22:4n-6 (open squares). The PKC activity for each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 μM. Three separate experiments for each fatty acid were performed, with triplicates for each concentration. See Figure 1 for abbreviations.

most of the *cis*-fatty acids followed by a decline in activity at 300 μM (Fig. 1). There was no significant difference between the maximum PKC activities attained in the presence of these different fatty acids. This suggested that the presence of a *cis*-double bond is essential for fatty acids to activate PKC and that the presence of additional double bonds in these fatty acids does not further affect their potency.

Comparison of the PKC-activating potency of three pairs of *cis*-fatty acids having the same number of double bonds but differing in chainlength, namely, palmitoleic acid (*c*-16:1n-7) and *cis*-vaccenic acid (*c*-18:1n-7), linolenic acid (18:3n-3) and docosatrienoic acid (22:3n-3), and arachidonic acid (20:4n-6) and adrenic acid (22:4n-6), suggested that there is no apparent correlation between the length of the chain and the potency of the *cis*-fatty acid (Fig. 2).

To define the effect of the configuration of the fatty acid double bond on the activation of PKC, four pairs of fatty acids were examined. Of four *trans*-fatty acids, namely palmitelaidic acid (*t*-16:1n-7), *trans*-vaccenic acid (*t*-18:1n-7), elaidic acid (*t*-18:1n-9), and linoelaidic acid (*t,t*-18:2n-6), all except *t,t*-18:2n-6 exhibited a much reduced ability to activate PKC compared to their corresponding *cis*-fatty acids, i.e., palmitoleic acid (*c*-16:1n-7), *cis*-vaccenic acid (*c*18:1n-7), oleic acid (*c*-18:1n-9) and linoleic acid (*c,c*-18:2n-6), respectively (Fig. 3). At the plateau concentration for *cis*-fatty acid (200 μM) or lower (50 and 100 μM), PKC activity in the presence of most *cis*-fatty acids was about twice that seen for the corresponding *trans*-fatty acids (Fig. 3). When the concentration was increased from 200 to 300 μM, most *cis*-fatty acids showed a slight decease in PKC activation, whereas *trans*-fatty acids exhibited a continuing increase in PKC activity. Even though the *trans*-fatty acids showed a different dose response from the *cis*-fatty acids, the maximum PKC activity cause by *trans*-fatty acids did not exceed the value attained by the corresponding *cis*-fatty acids within the dose range tested (10–300 μM). However, linoelaidic acid (*t,t*-18:2n-6), containing two *trans*-double bonds, caused a dose response and a maximum PKC activity similar to that of its *cis*-analog linoleic acid (*c,c*-18:2n-6) (Fig. 3).

A substituent on the chain markedly reduced the ability of *cis*-fatty acid to activate PKC. Two substituted fatty acids were chosen to examine the substituent effect, namely, 5,6-DHT, a dihydroxylated 20:3 *cis*-fatty acid which represents a 20:4n-6 with its C-5 double bond replaced by two hydroxyl substituted carbons, and 12,13-EODE, a 12(13)-epoxy oleic acid. Both substituted fatty acids gave the same dose-response curve with the maximum activity reaching approximately 50% of that caused by 20:4n-6 at 300 μM (Fig. 4).

Changes on the carboxyl group of a *cis*-fatty acid, such as the methyl ester of 18:3n-6 (Me-18:3n-6), completely abolished its ability to activate PKC (Fig. 4).

All HETE tested were capable of activating PKC at a dose of 100 μM or higher (Fig. 5). In general, all HETE

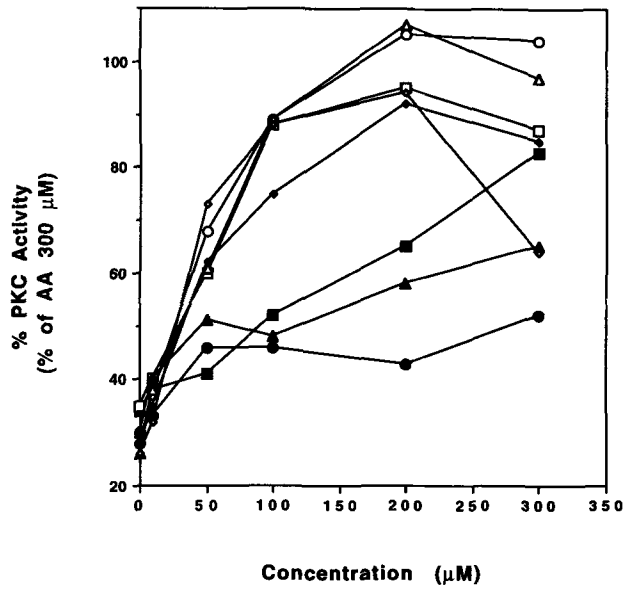

FIG. 3. Comparison of PKC activities induced by *cis*- or *trans*-fatty acids. Four pairs of *cis*- and *trans*-fatty acids were used: *c*-16:1n-7 (open squares), *t*-16:1n-7 (closed squares); *c*-18:1n-7 (open triangles), *t*-18:1n-7 (closed triangles); *c*-18:1n-9 (open circles), *t*-18:1n-9 (closed circles); *c,c*-18:2n-6 (open diamonds), *t,t*-18:2n-6 (closed diamonds). All *cis*-fatty acids are represented by open symbols; all *trans*-fatty acids are represented by closed symbols. The PKC activity for each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 μM. Three separate experiments for each fatty acid were performed, with triplicates for each concentration. See Figure 1 for abbreviations.

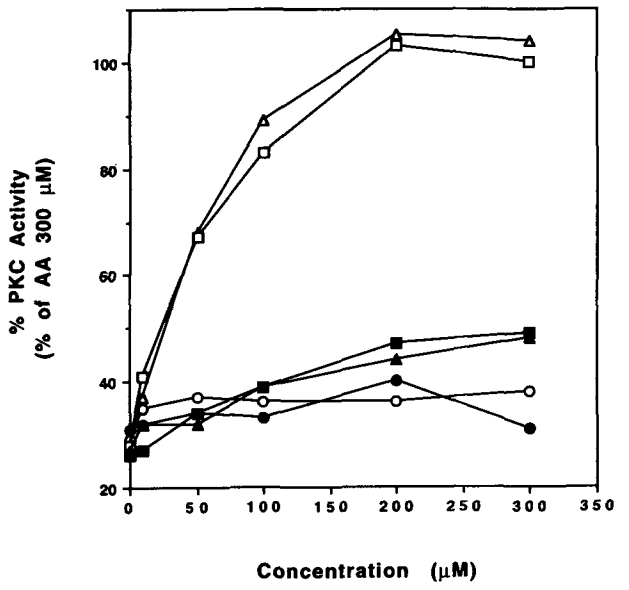

FIG. 4. Comparison of PKC activities induced by *cis*-fatty acids and their derivatives. Stearic acid (open circles), *c*-18:1n-9 (open triangles), 20:4n-6 (open squares), 5,6-dihydroxyeicosatrienoic acid, 20:3n-6 (closed squares), 12(13)-epoxyoctadienoic acid, 18:1n-9 (closed triangles) and Me-18:3n-6 (closed circles) were used. The PKC activity for each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 μM. Three separate experiments for each fatty acid were performed, with triplicates for each concentration. See Figure 1 for abbreviations.

isomers activated PKC to a similar extent, namely, between 150 and 200% of control values or between 35 and 55% of the activation caused by 20:4n-6 at 300 μM (Fig. 5). Although 9- and 13-HODE activated PKC at doses of 200 and 300 μM (Fig. 6), 9-HODE caused a higher maximal PKC activity than did the 13-HODE or 4 HETE isomers, which was about 60% of that induced by 20:4n-6 at 300 μM (Fig. 6). All hydroxylated metabolites of 20:4n-6 and *c,c*-18:2n-6 were capable of minimally activating PKC at concentrations above 100 μM.

DISCUSSION

In general, the potencies of the fatty acids and their derivatives to activate untreated mouse skin PKC can be ranked in decreasing order as follows: *cis*-fatty acids > *trans*-fatty acids ≥ substituted *cis*-fatty acids ≥ HETE and HODE > saturated fatty acids and methyl esters of *cis*-fatty acids.

Neither the saturated fatty acid 18:0 nor the methyl ester of a *cis*-fatty acid, Me-18:3n-6, activated PKC to any appreciable extent; both stimulated PKC less than 30% above control levels while 20:4n-6 at 300 μM resulted in an at least 300% increase over controls. These findings are in agreement with the results of studies done on rat brain (10,13). Seifert *et al.* (10) and Fan *et al.* (13) showed that 18:0 did not activate PKC in rat brain

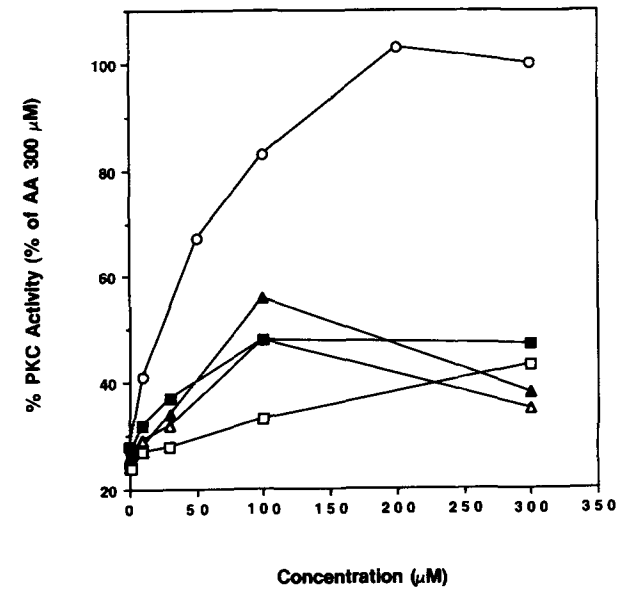

FIG. 5. Comparison of PKC activation induced by 20:4n-6 and its hydroxylated metabolites, hydroxyeicosatetraenoic acid (HETE). AA (20:4n-6) (open circles) and four HETE, i.e., 5(*S*)-HETE (closed triangles), 8(*S*)-HETE (open triangles), 12(*S*)-HETE (closed squares) and 15(*S*)-HETE (open squares) were used in this comparison. The PKC activity for each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 μM. See Figure 1 for other abbreviations.

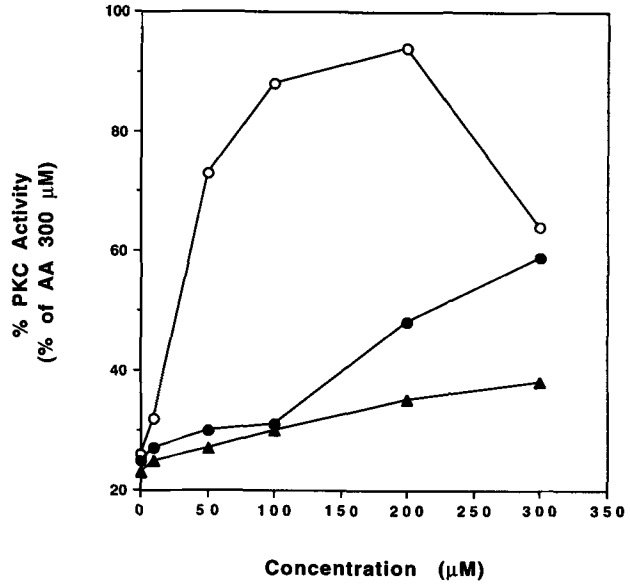

FIG. 6. Comparison of PKC activation induced by *c,c*-18:2n-6 and its hydroxylated metabolites, hydroxyoctadecadienoic acid (HODE). Linoleic acid (open circles) and two HODE, i.e., 9(*S*)-HODE (closed circles) and 13(*S*)-HODE (closed triangles), were used in the comparison. The PKC activity for each compound is expressed as the percentage of activity induced by 20:4n-6 at 300 µM. See Figure 1 for other abbreviations.

at 100 or 500 µM, respectively. Shinomura *et al.* (25) reported that at 50 µM concentration neither palmitic acid (16:0) nor 18:0 activated PKC in rat brain cytosol, whereas 20:4n-6 stimulated PKC activity to at least 200% of control levels. Seifert *et al.* (10) also showed that methyl arachidonate at 100 µM did not activate PKC while the parent fatty acid, 20:4n-6, stimulated PKC activity three- to fourfold above basal levels. Fan *et al.* (13) reported that methyl esters of *c*-18:1n-9, *c,c*-18:2n-6, 18:3n-3 and 20:4n-6 at 500 µM caused very little PKC activation (less than 20% of their parent fatty acids) in rat brain *in vitro* (13). Thus, at least one fatty acid double bond and the carboxyl group appear to be required to induce activation of PKC.

The number of double bonds and the chainlength of the fatty acids do not appear to be important factors in influencing activating potency, since most fatty acids containing one or more *cis*-double bonds gave virtually identical dose-response curves at concentrations between 10 and 200 µM. These findings are different from those reported by McPhail *et al.* (26) or Seifert *et al.* (10). McPhail *et al.* (26) indicated that the ability of fatty acids to activate PKC increased to some degree with an increase in the number of *cis* double bonds. The PKC activating potential of the three fatty acids studied by McPhail *et al.* (26) in decreasing order was found to be 18:3n-6 > *c,c*-18:2n-6 > *c*-18:1n-9. The dose response curves for *c,c*-18:2n-6 and *c*-18:1n-9 reported by McPhail *et al.* (26) were almost identical between 30 and 300 µM and the maximum PKC activity induced by 18:3n-6 was not much higher than that induced by either *c,c*-18:2n-6 or *c*-18:1n-9 in the presence of diolein. Also, 18:3n-6 appeared to be a less potent stimulator than *c,c*-18:2n-6 and *c*-18:1n-9 at concentrations other than the optimal one. Seifert *et al.* (10) reported that the maximum PKC activity induced by fatty acids increased with chainlength; however, among four *cis*-fatty acids, namely myristoleic acid (*c*-14:1n-5), *c*-16:1n-7, *c*-18:1n-7, *c*-18:1n-9, *c*-16:1n-7 induced the same maximum PKC activity as did *c*-18:1n-9, and *c*-16:1n-7 caused a higher PKC activity than either of the 18:1 fatty acids at 100 µM. The differences observed between different laboratories might be due to tissue-specific PKC isozyme distribution and activity. Studies on the activation of three types of PKC isozymes (α, β and γ) by fatty acids, including 20:4n-6, *c,c*-18:2n-6 and *c*-18:1n-9, clearly indicated isozyme specificity with regard to fatty acid-stimulated activation (27). The decreasing order of PKC activity induced by fatty acids for PKC α and β isozymes was 20:4n-6 > *c,c*-18:2n-6 > *c*-18:1n-9; however, the order was reversed with PKC γ. Wang *et al.* (5) demonstrated that mouse epidermal PKC isozymes exhibited different dose responses to treatment with 20:4n-6. They reported that PKC α, β, and δ showed one- to threefold increases in PKC activity when the concentration of 20:4n-6 was increased from 25 to 400 µM in the presence of PS and Ca^{2+}. PKC γ and ζ, on the other hand, exhibited high activity when stimulated with 20:4n-6 at 25 µM; at 400 µM concentration, 20:4n-6 almost completely inhibited the activity of PKC γ and ζ. Even though different potencies for *cis*-fatty acids were seen in rat brain in other studies and in mouse skin in our study, the effective dose ranges found in our study were in agreement with those observed by Seifert *et al.* (10) and McPhail *et al.* (26) (approximately 50 to 300 µM).

As discussed above, numerous studies have suggested that at least one *cis*-double bond is essential for activation of PKC. Leach and Blumberg (28) reported that saturated fatty acids such as butyric (4:0), lauric (12:0), myristic (14:0) and palmitic (16:0) acids showed very low ability to activate PKC, and they did not inhibit [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding activity in mouse brain cytosol (with the exception of lauric acid). On the other hand, *c,c*-18:2n-6, 18:3n-3 and 20:4n-6 markedly inhibited [^{3}H]PDBu binding in a concentration-dependent manner in the presence of phosphatidylcholine and PS. Leach and Blumberg (28) suggested that fatty acids may interact with PKC at a hydrophobic site which in turn allows the binding of [^{3}H]PDBu and the activation of PKC.

cis-Unsaturated fatty acids are known to perturb the lipid membrane and to increase membrane fluidity to a much greater extent than do saturated fatty acids or *trans*-unsaturated fatty acids (10,13,29,30). Although these rules cannot necessarily be applied to *in vitro* conditions, they suggest that configurational difference between *cis*- and *trans*-fatty acids could play a role in the ability of a fatty acid to interact with PKC. Seifert *et al.* (10) suggested that PKC activity stimulated by fatty acids might relate to fatty acid hydrophobicity and might not be due to a detergent effect. We found that fatty acids containing one *trans*-double bond exhibited

lower potencies than did the corresponding *cis*-fatty acids in activating PKC at the doses tested. These results were not in complete agreement with the observation made on rat brain by Seifert *et al.* (10) who found that *trans*-fatty acids containing 16 or 18 carbons, with the exception of *t*-18:1n-9, induced the same PKC activities as their *cis*-isomers. Ashendel *et al.* (31) had reported that PKC activity in mouse brain is much higher than in epidermis. Also, the differential distribution and activities of isozymes in tissues do probably contribute to the differences observed between brain and epidermal PKC activation.

The PKC activities induced by the two substituted *cis*-fatty acids, 5,6-DHT and 12,13-EODE, were even lower than those induced by *trans*-fatty acids. This gives further credence to the suggestion that the *cis*-configuration might be very important in the interaction between fatty acid and the hydrophobic site of PKC which would, in turn, modulate the PKC active site. The carbon chain of a *cis*-fatty acid exhibits a minimal 44° bend at the site of the double bond (32); this exposes the double bond far more than it does in a *trans*-fatty acid which essentially resembles a straight carbon chain. Substitution with a hydroxyl or epoxy group in the vicinity of the *cis*-double bond would provide stearic hindrance near the double bond. Thus, both *trans*-configuration and substitution close to the *cis*-double bond would reduce exposure of the double bond of the fatty acid and reduce interaction with PKC.

All HETE and HODE exhibited much lower potencies in activating PKC than did their parent *cis*-fatty acids, i.e., 20:4n-6 and *c,c*-18:2n-6, respectively. The hydroxylation of 20:4n-6 or *c,c*-18:2n-6 *via* lipoxygenase introduces one hydroxyl group at the site of one of the *cis*-double bonds; the *cis*-double bond is then shifted to the adjacent position and is converted into *trans*. The results of our study suggest that both the substituent effect and the configurational change make HETE and HODE much less potent PKC activators than are their parent fatty acids 20:4n-6 and *c,c*-18:2n-6. O'Brian *et al.* (11) reported that hydroperoxy metabolites of 20:4n-6 and *c,c*-18:2n-6, i.e., 15-hydroperoxyeicosatetraenoic acid (15-HpETE) and 13-hydroperoxyoctadecadienoic acid (13-HpODE), respectively, induced slightly reduced maximum activity but higher affinity to PKC than did 20:4n-6 or *c,c*-18:2n-6. On the other hand, 13-HODE exhibited both lower affinity and induced lower maximum activity. O'Brian *et al.* (11) suggest that oxidation of a component of PKC by hydroperoxy metabolites might be involved, rather than simply the binding to PKC. This would also explain why hydroperoxy metabolites of 20:4n-6 or *c,c*-18:2n-6 could possess the same structures as HETE and HODE yet exhibit potencies similar to those of their parent compounds.

In conclusion, the hydroxylated metabolites of 20:4n-6 and *c,c*-18:2n-6 produced *via* the lipoxygenase pathway were much less able to activate mouse skin PKC than were their parent compounds. Therefore, the ability of 20:4n-6 or *c,c*-18:2n-6 to activate PKC would be reduced once they were metabolized in the skin, and the possible role of 20:4n-6 or *c,c*-18:2n-6 in mouse skin tumor promotion *via* PKC activation would be less important. Furthermore, there was no significant difference in PKC activation between either 20:4n-6 and *c,c*-18:2n-6 or between HETE and HODE, which suggests that the inhibitory effect of *c,c*-18:2n-6 in mouse skin tumor promotion is not mediated by the activation of PKC by these fatty acids.

ACKNOWLEDGMENTS

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Lymphatic Absorption of Oxidized Cholesterol in Rats

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The absorption of cholesterol and of cholesterol oxidation products (oxidized cholesterols) was compared in lymph-cannulated rats. We found that the lymphatic absorption of an intragastrically administered, emulsified lipid meal containing 25 mg of cholesterol or 25 mg of oxidized cholesterols, within 24 h, was approximately 67 and 30%, respectively. The absorption rate of individual oxidized cholesterols differed considerably and was approximately 30% for 7α-hydroxycholesterol, 42% for 7β-hydroxycholesterol, 32% for 5β-epoxycholesterol, 28% for 5α-epoxycholesterol, 15% for cholestanetriol and 12% for 7-ketocholesterol. Moreover, cholesterol oxidation products delayed the absorption of oleic acid as triolein. Approximately 35 and 48% of cholesterol was recovered in chylomicrons (CM) and very low density lipoprotein (VLDL), respectively. In contrast, 54 and 40% of the oxidized cholesterols was recovered in CM and VLDL, respectively, although there was a significant difference in the distribution of individual oxidized cholesterols. The results of the present study indicate that oxidized cholesterols are absorbed to a lesser extent than is cholesterol, that they disturb fat absorption and that they distribute differently between lymphatic lipoproteins.
***Lipids* 29, 555–559 (1994).**

Cholesterol is a common component of most diets and undergoes autoxidation when exposed to heat, air, light and oxidizing agents. Processed foods are known to contain a variety of oxidized sterols including hydroperoxycholesterol, hydroxycholesterol, epoxycholesterol and ketocholesterol (1–6). Cholesterol oxidation products (oxidized cholesterols) exhibit diverse biological activities including cytotoxicity (7), mutagenicity (8), carcinogenicity (9) and atherogenicity (10). This has been concluded mainly from observations made *in vitro* whereas the *in vivo* effects of oxidized cholesterols are still less understood. Recently, Kendall *et al.* (11) demonstrated that dietary oxidized cholesterols moderately enhance colon carcinogenesis when compared to cholesterol in mice, and Berezov *et al.* (12) reported on the immunodepressive activity of oxidized cholesterols. To better appreciate the physiological effects of oxidized cholesterols, it is necessary to understand the extent and the mode of their absorption from the intestine. At present, only minimal information is available in regard to the absorption of oxidized cholesterols (13–16).

In the present study, the pattern of absorption of oxidized cholesterols and their effect on fat absorption were investigated in lymph-cannulated rats. The distribution of oxidized cholesterols between lymph lipoproteins was also determined.

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; CM, chylomicron; EDTA, ethylenediaminetetraacetic acid, disodium salt; GC, gas chromatography, HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. Cholesterol [purity, 99.7% by gas chromatography (GC); no oxidized cholesterols were detected] and sodium taurocholate (purity > 98%) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Triolein (purity > 99%), pentadecanoic acid and 5α-cholestane were from Sigma Chemical Co. (St. Louis, MO). Fatty acid-free bovine serum albumin was from Miles Inc. (Kankakee, IL).

Preparation of cholesterol oxidation products. Cholesterol was melted and kept in an electric oven at 150°C for 12 h. The oxidized cholesterols formed (17) were then applied onto a silicic acid column (24 mm × 700 mm; silica gel 60, 70–230 mesh; E. Merck, Darmstadt, Germany) and fractionated by successive elution with 200 mL of *n*-hexane, 200 mL of diethyl ether, and finally 300 mL of methanol. An aliquot of the polar fraction eluted with methanol was dried on an rotary evaporator and then *in vacuo*. The composition of the oxidized cholesterol-rich fraction thus obtained is shown in Table 1. The preparation consisted of more than 85% oxidized cholesterols.

Animal treatment. Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 300–340 g were given a commercial nonpurified diet (type NMF, Oriental Yeast Co., Tokyo, Japan) for 2 d after arrival and drinking water *ad libitum*. Rats under nembutal anesthesia were subjected to cannulation of the left thoracic lymph channel, and an indwelling catheter was placed in the stomach (18). An osmotically normal solution containing 139 mM glucose and 85 mM NaCl was infused continuously during lymph collection at a rate of 3 mL/h through the gastric tube (19). After surgery, animals were placed in restraining cages in a warm recovery room and allowed free access to drinking water containing 139 mM glucose and 85 mM NaCl. On the next morning, after collecting lymph for 2 h (blank lymph) to

TABLE 1

Composition of Cholesterol Oxidation Products

Sterols	Weight %
7α-Hydroxycholesterol	8.8
Cholesterol	12.0
7β-Hydroxycholesterol	8.2
5β-Epoxycholesterol	7.1
5α-Epoxycholesterol	6.0
Cholestanetriol	4.7
7-Ketocholesterol	30.2
Unknowns[a]	23.0

[a]Composed of more than 20 components.

determine the amounts of endogenous cholesterol and oleic acid secreted per hour in lymph, each animal was administered 3 mL of a test emulsion through the gastric tube, and the lymph was collected for 24 h beginning at 10:00 a.m. The 3-mL test emulsion contained 200 mg of sodium taurocholate, 50 mg of fatty acid-free albumin, 200 mg of triolein and 25 mg of either cholesterol or cholesterol oxidation products. The emulsions were prepared by sonication at room temperature using an Ultrasonic Disruptor Model UR-200P (Tomy Seiko Co. Ltd., Tokyo, Japan). The lymph was collected in tubes containing ethylenediaminetetraacetic acid disodium salt (EDTA; 50 mg) for 3-h periods during the first 9 h after administration of the test emulsion and in a single collection from 9–24 h thereafter.

Lipids analysis. Lymph lipids were extracted with 20 vol of chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (20), and then saponified in ethanolic 1 N KOH at 70°C for 1 h (21). Although some studies (22,23) have suggested that hot alkaline saponification can decompose oxidized cholesterols, the standards we used were stable under the conditions employed. The unsaponifiable fraction was extracted with *n*-hexane and converted to trimethylsilyl ethers by reaction with a mixture of trimethylchlorosilane, 1,1,1,3,3,3-hexamethyldisilazane and anhydrous pyridine (1:3:9, by vol) at room temperature. The trimethylsilyl ethers were separated by capillary GC using 5α-cholestane as internal standard. The fatty acid composition was determined by GC of the methyl esters that were prepared by transmethylation of the lipids extracted with BF_3/methanol in the presence of pentadecanoic acid as internal standard (24). Total and free sterols were measured by the method of Sperry and Webb (25). Free and esterified cholesterols were separated by thin-layer chromatography (silica gel 60, 0.25 mm thick layers; E. Merck) using hexane/diethyl ether (60:40, vol/vol) as developing solvent. The sterols were eluted with methanol and analyzed by capillary GC as described above.

The absorption rates of each of the sterols and of triolein were determined by subtracting the amounts of sterols and oleic acid in blank lymph from those found in the experimental lymph, and the resulting values were divided by the amounts of sterols or oleic acid administered to obtain absorption rates.

Isolation of lymph lipoproteins. Lymph collected in ice-cooled tubes was separated into the major lipoprotein fractions by ultracentrifugation (26). Chylomicrons (d < 1.006 g/mL) were collected at 23,000 × *g* for 40 min and very low density lipoprotein (VLDL) (d < 1.006 g/mL) at 50,000 × *g* for 16 h.

GC. Trimethylsilyl derivatives of oxidized cholesterols in lymph were separated by capillary GC (GC-7AG; Shimadzu Co., Kyoto, Japan) using a flame-ionization detector, a fused silica capillary ULBON HR-1 column with a liquid phase thickness of 0.25 μm (0.25 mm × 50 m; Shinwa Chemical Industries, Kyoto, Japan), and a Shimadzu C-R6A integrator. The oven and injector temperatures were 280 and 300°C, respectively, and the helium flow rate was 2.2 mL/min. Individual oxidized cholesterols were identified as described previously (27). Methyl esters of fatty acids were analyzed using a Shimadzu gas chromatograph (GC-8A) equipped with a flame-ionization detector, a 10% Silar 10C column (3 mm × 2 m), and a C-R5A integrator. The oven and injector temperatures were 210 and 250°C, respectively. The flow rate of nitrogen gas was 25 mL/min.

Statistical analysis. Duncan's new multiple-range test (28) and Student's *t*-test (29) were used to define the differences between values.

RESULTS

There were significant differences in lymph flow rates between the rats that received cholesterol and of those that received the oxidized cholesterols, and the flow rates were significantly lower in the latter (Table 2).

Figure 1 shows the chromatographic patterns of the cholesterol oxidation products administered and of those recovered in lymph. Figure 2 illustrates the lymphatic absorption of cholesterol and of the various oxidized cholesterols. As can be seen, cholesterol was absorbed within 24 h at a higher rate than were the oxidized cholesterols, i.e., 66.8 ± 4.5% vs. 30.0 ± 2.6%, respectively. However, the rate of absorption of cholesterol in the presence of the cholesterol oxidation products was significantly lower than that in rats that received cholesterol alone (50.9 ± 4.2% vs. 66.8 ± 4.5%, respectively). The absorption rates within 24 h of cholestanetriol (15.2 ± 3.6%) and 7-ketocholesterol (12.0 ± 2.6%), in turn, were significantly lower than those of the other oxidized cholesterols, while those of 7α-hydroxycholesterol, 5α-epoxycholesterol and 5β-epoxycholesterol were quite similar (30.4 ± 3.2, 27.5 ±2.5 and 32.3 ± 6.7%, respectively). 7β-Hydroxycholesterol (41.5 ± 5.6% 24 h) was absorbed at a higher rate than were the other oxidized cholesterols.

As shown in Figure 3, the cholesterol oxidation products interfered with the lymphatic absorption of oleic acid

TABLE 2

Body Weight and Lymph Flow[a]

Groups	Body weight (g)	Lymph flow (mL) 0–3 h	3–6 h	6–9 h	9–24 h	0–24 h
Cholesterol	332 ± 16	25.8 ± 1.4[b]	24.1 ± 2.4[b]	24.4 ± 2.0[b]	102 ± 11[b]	176 ± 16[b]
Cholesterol oxidation products	320 ± 3	10.8 ± 1.4	8.9 ± 1.0	9.1 ± 0.8	42.6 ± 2.2	71.4 ± 4.0

[a]Values are means ± SE of 5–7 rats. [b]Significantly different from the corresponding cholesterol oxidation products group at $P < 0.001$ (Student's *t*-test).

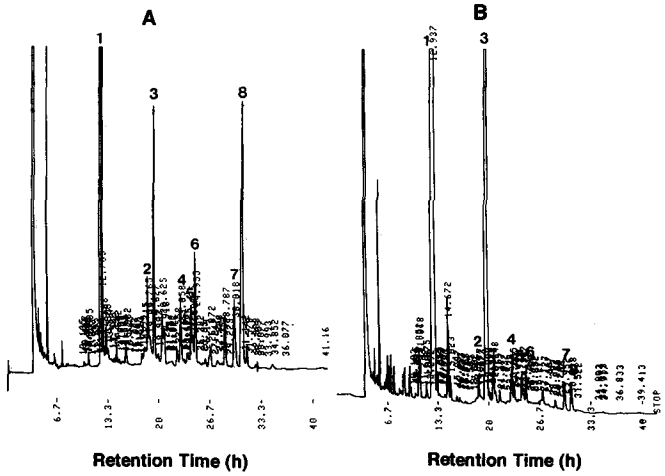

FIG. 1. Gas chromatography of the cholesterol oxidation products administered (A) and of the oxidized cholesterols recovered in lymph (B). Peaks: 1, 5α-cholestane (internal standard); 2, 7α-hydroxycholesterol; 3, cholesterol; 4, 7β-hydroxycholesterol; 5, 5β-epoxycholesterol; 6, 5α-epoxycholesterol; 7, cholestanetriol; and 8, 7-ketocholesterol.

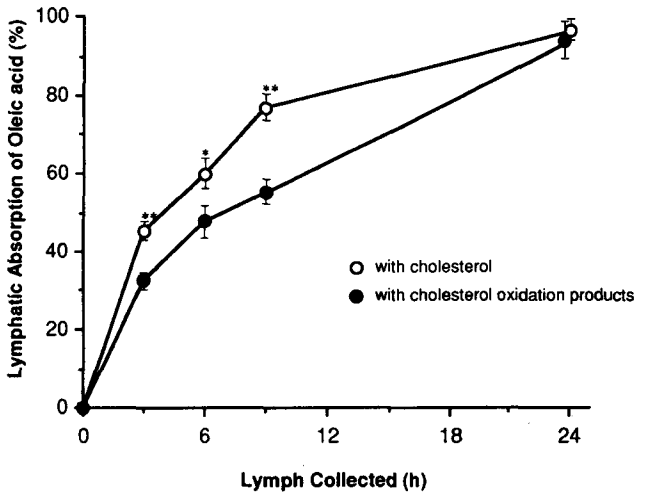

FIG. 3. Lymphatic absorption of oleic acid administered as triolein. Thoracic duct lymph was cannulated as described in the Materials and Methods section and cholesterol or cholesterol oxidation products was administered by stomach catheter. Lymph was collected periodically for 24 h. The percentage shows the rate of the oleic acid recovered in lymph to the oleic acid administered in the test emulsion. Values are means ± SE for five rats. Values are significantly different for the corresponding oxidized cholesterol group at *$P < 0.05$ and **$P < 0.01$ (Student's t-test).

when administered as triolein during the first 6 h after administration, but the difference disappeared after 24 h (96.3 ± 5.7 and 95.8 ± 5.2% for the cholesterol and the cholesterol oxidation products groups, respectively).

The distribution of cholesterol and of oxidized cholesterols between lymph lipoproteins is shown in Figure 4. A differential distribution between lymph lipoproteins is seen between cholesterol and oxidized cholesterols. Approximately 35% of cholesterol was carried in chylomicrons (CM) while 48% was associated with VLDL. In contrast, more oxidized cholesterols than cholesterol were generally associated with CM, whereas the levels associ-

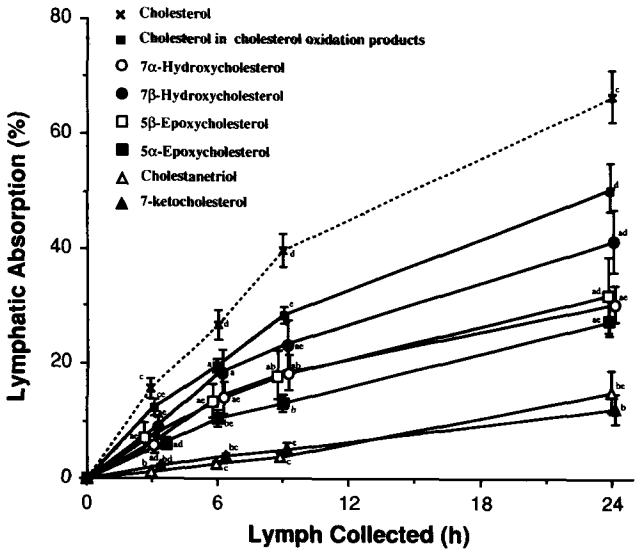

FIG. 2. Lymphatic absorption of cholesterol and of various oxidized cholesterols. Thoracic duet lymph was cannulated as described in the Materials and Methods section and cholesterol or the cholesterol oxidation products was administered by stomach catheter. Lymph was collected periodically for 24 h. Absorption in percentage is the percentage of the dose. Values are means ± SE for five rats. Values at each point not sharing a common letter are significantly different at $P < 0.05$ (Duncan's new multiple-range test).

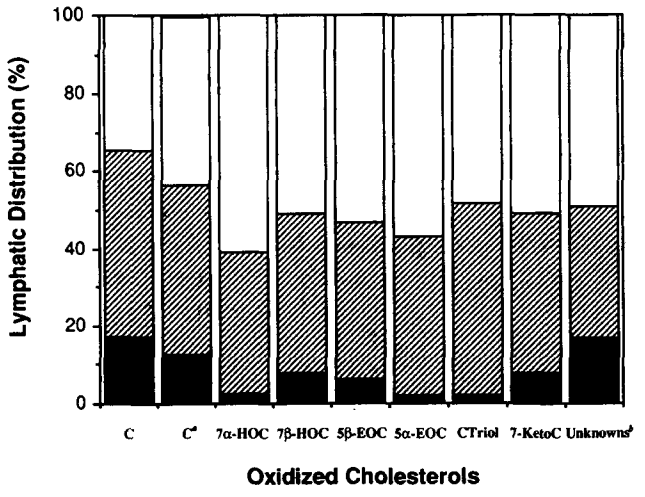

FIG. 4. Distribution of cholesterol and oxidized cholesterols between lymph lipoproteins. Lymph lipoproteins were separated as described in the Materials and Methods section. Open bar represents chylomicrons, hatched bar represents very low density lipoprotein, closed portion of bar represents bottom fraction (d < 0.06). C, cholesterol; 7α-HOC, 7α-hydroxycholesterol; 7β-HOC, 7β-hydroxycholesterol; 5β-EOC, 5β-epoxycholesterol; 5α-EOC, 5α-epoxycholesterol; CTriol, cholestanetriol; 7-ketoC, 7-ketocholesterol. [a]Cholesterol in cholesterol oxidation products. [b]Unknowns are described in Table 1.

TABLE 3

Oxidized Cholesterols in Esterified and Nonesterified Sterol Fractions of Lymph[a]

Sterols	Sterol fraction	
	Esterified	Nonesterified
	(weight %)	
7α-Hydroxycholesterol	1.1 ± 0.3[c]	4.3 ± 0.3
Cholesterol	80.7 ± 1.6[c]	54.5 ± 1.5
7β-Hydroxycholesterol	1.6 ± 0.4[c]	14.5 ± 0.5
5β-Epoxycholesterol	2.1 ± 0.3	2.5 ± 0.7
5α-Epoxycholesterol	5.1 ± 0.3[c]	3.0 ± 0.7
Cholestanetriol	0.2 ± 0.1[c]	1.5 ± 0.2
7-Ketocholesterol	2.3 ± 0.1[c]	1.4 ± 0.1
Unknowns[b]	1.2 ± 0.5[c]	18.1 ± 0.8

[a]Values are means ± SE of five rats.
[b]Composed of more than 20 compounds.
[c]Significantly different from the corresponding nonesterified fraction at $P < 0.05$ (Student's *t*-test).

ated with VLDL tended to be low except for cholestanetriol. Lesser amounts of oxidized cholesterols than of cholesterol were present in lipoprotein fractions heavier than CM and VLDL.

The degree of sterol esterification in rats given cholesterol oxidation products was lower than in those given cholesterol (68.4 ± 0.9 and 79.6 ± 6.0% for oxidized cholesterols and cholesterol, respectively). The compositions of cholesterol and of the oxidized cholesterols in the nonesterified and esterified sterol fractions of lymph are shown in Table 3. The esterified sterol fraction contained about 81% cholesterol while the free sterol fraction contained approximately 55% cholesterol. The oxidized cholesterols were also not evenly distributed between the esterified and the nonesterified sterol fractions. Thus, for example, considerably higher amounts of 7β-hydroxycholesterol were present in the free rather than the esterified sterol form.

DISCUSSION

Oxidized cholesterol derivatives occur in many food products. They have been identified in butter (1), dairy products (2), egg products (1,3), heated tallow (4), pancakes (5), French fries (5) and various meat products (6). We also showed that oxidized cholesterols are present in processed marine foods (30). The *in vivo* absorption of oxidized cholesterols is still not very well understood. Peng *et al.* (13) have previously analyzed the composition of various cholesterol oxidation products in serum after gastric administration to rabbits, and they assumed that the rate of absorption of oxidized cholesterols is not significantly different from that of cholesterol. Bascoul *et al.* (14) have shown that 5α-epoxycholesterol after oral administration to rats was largely absorbed (about 90%) by the intestinal mucosa. Moreover, available data on the absorption of oxidized cholesterols have shown that their absorption appears to be similar to that of cholesterol based on tracer studies and that dietary oxysterols are absorbed from the gastrointestinal tract quite rapidly (16,31). The present study suggests that considerable differences exist in the degree of absorption of various oxidized cholesterols, and that less of the oxidized cholesterols was absorbed than of the cholesterol administered.

Several mechanisms could explain the lower rates of absorption of oxidized cholesterols. Firstly, it is possible that the oxidized cholesterols are less soluble as micelles than is cholesterol, as it has been shown that lower micellar solubility results in lower cholesterol absorption (17,32). The higher polarity of the oxidized cholesterols may, indeed, interfere with micelle formation. We found that lipid emulsions containing the cholesterol oxidation products were much less stable than those containing cholesterol, and precipitates formed when such emulsions were kept at room temperature. Secondly, it would also be possible that the lower degree of lymphatic absorption of oxidized cholesterols could be attributed to their lesser susceptibility to esterification in the small intestinal mucosal cells. Esterification is a mandatory step in the sterol absorption process. Thirdly, since oxidized cholesterols can exert strongly cytotoxic effects (7), they may injure the mucosal cells, which could explain the reduced lymph flow in rats given cholesterol oxidation products. Reduced lymph flow, in turn, may reduce the absorption of oxidized cholesterols. However, the lymph flow rates and absorption rates did not necessarily parallel each other in the present study, suggesting that the reduced flow rate may only be a contributing cause of the observed differences.

Most of oxidized cholesterols appeared in the nonesterified sterol fraction of lymph. This may be one of the reasons for the poor absorption of the oxidized cholesterols as has previously been shown for ß-sitosterol, which is a poorly absorbable plant sterol (33). Although our results would suggest inhibition by oxidized cholesterols of the acyl coenzyme A:cholesterol acyltransferase (ACAT) reaction, it has been shown in cell culture studies (34) that ACAT activity is actually stimulated by oxidized cholesterols. Nevertheless, the lower rate of esterification of oxidized sterols and their poor absorption may still be due to the fact that oxidized cholesterols serve as structurally poor substrates for the esterification reaction because of the presence of hindering hydroxy-, keto- and epoxy groups.

Our study also showed that cholesterol oxidation products interfered with the lymphatic absorption of cholesterol and of oleic acid given as triolein (Figs. 2 and 3). The mechanisms of these effects are presently unknown.

Furthermore, the distribution of the oxidized cholesterols between the lymphatic lipoproteins was found different than that of cholesterol. Ikeda *et al.* (33) had shown that both β-sitosterol and cholesterol were exclusively associated with CM. Since more of the oxidized cholesterols were carried in CM than was cholesterol (Fig. 4), their metabolic fate may also differ. Peng *et al.* (13,35) had reported that cholestanetriol, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol showed a preference for being transported in VLDL, whereas most of the 25-hydroxycholesterol was associated with low density lipoprotein (LDL) of the sera of rabbits or squirrel monkeys. Also, Brooks *et al.* (36) had found that, for example, α- and β-epoxides of cholesterol, 7-ketocholesterol and 26-hydroxycholesterol were predominantly carried by VLDL and LDL in serum of type II hypercholesterolemic pa-

tients. Moreover, Emanuel *et al.* (15) had reported that some oxidized cholesterols may be selectively absorbed and transported *via* CM. Thus, oxidized cholesterols are predominantly distributed in the serum lipoproteins of lower densities including CM, VLDL and LDL, whereas only minute amounts are associated with high density lipoproteins (HDL). Oxidized cholesterols have been found in human aortic tissues and plaques (37), and the selective transport of oxidized cholesterols by CM, VLDL and LDL may contribute in part to their atherogenicity (10).

Oxidized cholesterols have been implicated in the initiation and/or progression of atherosclerosis due to their toxicity to arterial endothelial and smooth muscle cells both *in vitro* (38) and *in vivo* (39). The presence of oxidized cholesterols in oxidized human LDL has been reported (40). Also Baranowski *et al.* (41) reported that oxidized cholesterols including 25-hydroxycholesterol, 5β-epoxycholesterol and cholestanetriol promoted tissue inflammation and necrosis and also caused cell death in cultures. Since a relatively large amount of oxidized cholesterols was absorbed from the intestine, they may exert various secondary effects *in vivo*.

In conclusion, the present study has demonstrated that lymphatic absorption of oxidized cholesterols occurs to a considerable degree in rats. Approximately 30% of the oxidized cholesterols was absorbed. Nakatsugawa and Kaneda (42,43) had shown that 0.23 and 0.5–0.6% of the methyl linoleate hydroperoxides administered were found in the lymph of rabbits and rats in unchanged form, respectively. Thus, oxidized cholesterols appear to be absorbed more readily than are autoxidized fatty acids. The oxidized cholesterols used in this study may not be altogether like naturally autoxidized cholesterol present in foods. However, our preceding study (30) had shown that the composition of oxidized cholesterols in fish products did not differ depending on the source. Since cholesterol in foods is quite easily oxidized when oxidizable fats are present, particularly during high-temperature cooking (30), more attention should be paid to oxidized cholesterols present in the diet. In fact, the concentration of oxidized cholesterols was found increased in human plasma CM after consumption of a powdered egg meal containing 30–90 ppm oxidized cholesterols (15). Also, Jacobson (44) inferred that dietary oxidized cholesterols from ghee may be responsible for the high frequency of atherosclerotic complications in Indian immigrant populations. More studies are needed to elucidate the effect of various dietary cholesterol oxidation products on metabolic processes.

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Effects of n-3 and n-6 Fatty Acids on the Activities and Expression of Hepatic Antioxidant Enzymes in Autoimmune-Prone NZB×NZW F_1 Mice

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Menhaden fish oil (FO) containing n-3 fatty acids dramatically extends the life span and delays the onset and progression of autoimmune disease in (NZB×NZW)F_1 (B/W) female mice as compared to those fed corn oil (CO) rich in n-6 lipids. As an inefficient antioxidant defense system has been linked to autoimmune diseases, the present study was undertaken to determine whether the protective action of n-3 lipids is mediated through their antioxidant defense system. Weanling B/W mice were fed a nutritionally adequate, semipurified diet containing CO or krill oil (KO) or FO at 10% level (w/w) *ad libitum* until the mice were 6.5 months old. All diets contained the same level of vitamin E (21.5 mg/100 g diet). We compared the effects of feeding n-6 and n-3 lipids on survival, kidney disease, hepatic microsomal lipid composition, peroxidation, and on the activity and mRNA expression of the antioxidant enzymes catalase, glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in 6.5-month-old B/W mice. The results showed that when compared to livers from CO-fed mice, livers from KO- and FO-fed mice showed: (i) significantly higher ($P < 0.001$) activities and expression of CAT, GSH-Px and SOD; (ii) significantly lower ($P < 0.001$) arachidonic acid (20:4n-6) and linoleic acid (18:2n-6) and higher ($P < 0.001$) eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) levels in hepatic microsomes; and (iii) significantly lower ($P < 0.001$) estimated peroxidation indices and thiobarbituric acid reactive substances generation. The data indicate that one of the mechanisms through which the n-3 lipids delay the onset of autoimmune diseases in B/W mice may be through maintenance of higher activities and expression of hepatic antioxidant enzymes.

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Autoimmune-prone (NZB×NZW)F_1 (B/W) female mice spontaneously develop severe lupus nephritis as they age. At an early age they also exhibit vascular lesions resembling systemic lupus erythematosus (SLE) in humans, and these mice die between 6 and 12 months of age (1). Earlier studies had shown that caloric restriction and the source of dietary fat can have a profound effect on autoimmune SLE in mice (2–5). Our ongoing studies have indicated that n-3 fatty acid-rich menhaden fish oil (FO) dramatically extends the life span and delays the onset of autoimmune disease, whereas corn oil (CO), rich in n-6 fatty acids, significantly shortens the life span and accelerates autoimmune processes in these mice (6,7). We also observed that feeding *ad libitum* a diet containing 20% FO and/or moderate caloric restriction inhibited autoimmune disease in these mice. The beneficial effects observed have been primarily attributed to the increased eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) contents and to the markedly decreased 20:4n-6 levels in the FO-fed animals.

The deleterious effects of oxidative stress have previously been associated with various pathological conditions (8–10), but the role of an impaired or defective antioxidant defense system in autoimmune diseases has been explored only to a limited extent (11–13). Our recent study on MRL/*lpr* mice indicated that along with other immunological abnormalities, these mice clearly showed a defective antioxidant defense system as the expression of hepatic antioxidant enzymes was significantly lower when compared to that of an MRL/+ strain (14). It is not yet known, however, whether the n-3 dietary lipids of FO, which are known to ameliorate autoimmune disease, are also able to modulate the liver antioxidant defense system in animals prone to developing autoimmune disease. The susceptibility of an organism to oxidative damage is affected by the efficiency of the antioxidative defense system to cope with the oxidative stress which, in turn, can be influenced by nutritional intervention with antioxidants (15). Antioxidant enzymes, such as glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD), as well as antioxidant nutrients, such as vitamin E, vitamin A, vitamin C and selenium, can participate in controlling lipid peroxidation (16–18). Because antioxidant enzymes play an important role in the protection against free radical damage, a decrease in the activities or expression of these enzymes may predispose tissues to free radical injury (19,20).

The present study was undertaken to compare the effects of dietary CO, krill oil (KO) and FO on hepatic microsomal lipid composition, peroxidation and the expression of mRNA for three antioxidant enzymes (CAT, GSH-Px and SOD) in B/W female mice. In addition, we also tested crude krill lecithin (KO) as an alternate source of n-3 lipids. The data we obtained indicate that besides having a decreased survival rate, the CO-fed B/W mice exhibited lower activities and lower mRNA expression of the three antioxidant enzymes, as well as higher levels of 20:4n-6, and increased peroxidation in liver tissue. Our

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Abbreviations: B/W mice, (NZB×NZW) F_1 mice; CAT, catalase; CO, corn oil; EDTA, ethylenediaminetetraacetic acid; FO, fish oil; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione reduced form; GSH-Px, glutathione peroxidase; KO, krill oil; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; SDS, sodium dodecylsulfate; SLE, systemic lupus erythematosus; SOD, superoxide dismutase; SSC, sodium chloride/sodium citrate buffer; TBARS, thiobarbituric acid reactive substances.

present data also show that with adequate antioxidant supplements, FO can restore or enhance the antioxidant defense system and prolong the life span of autoimmune prone B/W mice.

MATERIALS AND METHODS

Experimental animals and diets. B/W female mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The weanling mice (20/group; 15 mice for survival study and 5 mice/group for cross sectional study at 6.5 months) were fed *ad libitum* nutritionally adequate, semipurified diets prepared weekly containing 10% (w/w) CO (ICN, Irvine, CA), crude KO (a generous gift from Itano Refrigerated Food Co., Ltd., Tokushima, Japan) or odor-free FO (U.S. Department of Commerce, National Marine Fisheries Service, Charleston, NC). The fatty acid composition of the three oils is given in Table 1. The current AIN diet formula contains the following diet ingredients: casein, 20%; dextrose, 30%; starch, 31%; oil, 10%; cellulose, 3.5%; AIN salt mixture, 3.5%; AIN vitamin mixture, 1.5%; DL-methionine, 0.3%; and choline, 0.2%. All three oils had equal levels of antioxidants, i.e., 1.3 g (1300 I.U.) α-tocopherol per kg oil, 1.2 g (13.2 I.U.) γ-tocopherol/kg and 1 g/kg *tert*-butylhydroquinone, as recommended by the National Institutes of Health (NIH) to prevent peroxidation during storage. The FO and KO diets also contained 1% CO to meet essential fatty acid requirements. The final concentration of vitamin E (DL-α-tocopheryl acetate, γ-tocopherol and α-tocopherol) was ~215 I.U./kg diet. Fresh food was provided daily in the evening, and precautions were taken to prevent oxidation of lipids and diets. The mice were maintained in plastic cages under a 12-h light/dark cycle. The body weights and proteinuria were recorded once a month. NIH guidelines were strictly followed. The animals (5 mice/group) were sacrificed at 6.5 months of age by cervical dislocation, livers were collected in ice-cold physiological saline, and part of the livers was frozen in liquid nitrogen and stored at –80°C for RNA isolation.

TABLE 1

Fatty Acid Composition of the Oils[a]

Fatty acid	Corn oil (%)	Fish oil (%)	Krill oil (%)
14:0	0.03	8.40	9.67
16:0	10.33	13.97	17.00
18:0	1.99	2.51	1.07
20:0	—	0.14	0.07
16:1	0.16	11.17	6.40
18:1	24.00	10.00	15.40
18:2n-6	52.60	0.66	1.64
18:3	0.09		
20:4n-6	—	1.45	0.52
20:5n-3	—	13.37	11.14
22:4n-6	—	0.11	—
22:5n-6	—	0.39	—
22:5n-3	—	2.06	0.38
22:6n-3	—	8.60	6.77
Others	10.80	27.20	29.40

[a]The analysis was carried out by the U.S. Department of Commerce, NOAA, National Marine and Fisheries Service, Southeast Fisheries Center, Charleston Laboratory (Charleston, SC).

Preparation of liver cytosol and microsomes. The livers were rinsed in ice-cold physiological saline and minced with scissors. A 10% homogenate of livers was prepared in 0.15 M KCl buffer (pH 7.4) and centrifuged at 12,000 × *g* for 10 min. The pellet was discarded, and the supernatant was collected and centrifuged at 105,000 × *g* for 90 min. The supernatant was the cytosolic fraction that was saved for determining the activities of the antioxidant enzymes. The pellet was resuspended in the buffer and centrifuged at 12,000 × *g* for 15 min to wash and collect the microsomal pellet. The microsomal pellets then were resuspended in the homogenization buffer. The protein content of the microsomes was measured using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Fatty acid analyses and lipid peroxidation analyses were carried out on the liver microsomal fractions.

Measurement of thiobarbituric acid reactive substances (TBARS). TBARS in liver homogenates were measured using a modification of the thiobarbituric acid assay by Laganiere and Yu (21). A 10-min centrifugation at 2000 × *g* replaced the lengthy organic solvent extraction of pigments. Malondialdehyde (as tetraethyl acetal; Aldrich Chemical Company, Milwaukee, WI) was used as standard.

Fatty acid analyses of liver microsomal total lipids. Lipids from liver microsomes were extracted with chloroform/methanol (2:1, vol/vol). Fatty acid methyl esters were obtained by transesterification with borontrifluoride/methanol reagent (90°C, 2 h) as described previously (22). The methyl esters were extracted with hexane and analyzed on a Perkin Elmer (San Jose, CA) 8420 gas chromatograph equipped with a fused silica capillary column (22). Chromatographic conditions included an oven temperature of 205°C, a flame-ionization detector set at 250°C, and helium as carrier gas at a flow rate of 0.9 mL/min. Mixtures of fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used for peak identification.

Expression of hepatic antioxidant enzymes (CAT, GSH-Px, SOD) mRNA. Total RNA was isolated from the frozen livers using the acid/guanidinium isothiocyanate/phenol/chloroform extraction procedure (23), and the RNA was quantified spectrophotometrically. Twenty micrograms of total RNA was electrophoresed in 0.8% agarose/2.2 M formaldehyde gel. The gel contained ethidium bromide (0.5 μg/mL) in order to verify RNA integrity and loading equivalency. RNA was electroblotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) and fixed to the membrane by cross-linking, using Stratagene UV crosslinker-1800 (Stratagene, La Jolla, CA). The blots were prehybridized, hybridized and then washed following the procedure described in detail by Korc *et al.* (24). In brief, the blots were prehybridized overnight at 42°C in a buffer containing 50% formamide/0.1% sodium dodecylsulfate (SDS)/0.5X sodium chloride/sodium citrate buffer (SSC) (1X SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/2X Denhardt's (1X Denhardt's = 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin)/250 μg/mL sonicated, denatured salmon sperm DNA and

50 mM sodium phosphate, pH 6.5. The blots were then hybridized at 42°C for 18 h in the same buffer to which 10% dextran sulfate and the ^{32}P-labeled cDNA probe (1×10^6 cpm/mL) were added. The blots were then washed at 23°C three times for 5 min each in a buffer containing 2X SSC/1.0% SDS, followed by three washes in 0.1X SSC/1% SDS (high stringency). The blots were exposed at –80°C to Kodak XAR-5 film with Dupont intensifying screens. The intensity of the autoradiographic bands was semi-quantified by videoimage analysis using NIH Image 1.4 program, and the values were expressed in fold change as a ratio of the densitometric value of CAT, GSH-Px and SOD to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control. The size of mRNA was determined by its relative positions to that of ethidium bromide-stained 28S and 18S ribosomal RNA and 0.24-9.5 kb RNA ladder (Gibco-BRL, Grand Island, NY). The cDNA used in this study were CAT (1.3 kb *Bam* HI/*Sph*I fragment) (25), GSH-Px (0.55 kb *Eco*RI fragment) (26), SOD (0.65 kb *Pst* I fragment) (27) and GAPDH (1.0 kb *Bam* H1-*Pst* I fragment) (28). The cDNA probes (CAT, SOD, GAPDH) were obtained from American Type Culture Collection (Rockville, MD). The cDNA were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL) by random prime labeling (Boehringer Mannheim, Indianapolis, IN) to obtain a specific activity of $0.8–1.2 \times 10^9$ cpm/μg.

Measurement of SOD, GSH-Px and CAT enzyme activities in the cytosolic fractions. The chemicals used in the enzyme assays were obtained from Sigma Chemical Company (St. Louis, MO). All three enzyme activities in the cytosolic fraction of livers were measured in a Shimadzu UV-Visible recording spectrophotometer UV-265 (Shimadzu Scientific Instruments, Houston, TX). CAT (EC 1.11.1.6) activity was measured by monitoring the decomposition of H_2O_2 at 240 nm as described by Aebi (29). The reaction mixture (3 mL) consisted of 50 mM phosphate buffer (pH 7.0) and 1 mL 30 mM H_2O_2 in phosphate buffer and suitably diluted cytosol. CAT activity was expressed as μmoles of hydrogen peroxide reduced/min/mg protein. GSH-Px (EC 1.11.1.9) activity was measured using the coupled-enzyme system as previously described (30). The reaction mixture consisted of 0.5 mL potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mL of 10 mM GSH and 0.1 mL of 2.4 U/mL glutathione reductase. The mixture was preincubated for 10 min at 37°C. Thereafter, 0.1 mL of 1.5 mM β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) was added, and hydroperoxide-independent consumption of NADPH was monitored for three min. The overall reaction was started by adding 0.1 mL of 12 mM *t*-butyl hydroperoxide solution, and the decrease in absorption at 340 nm was monitored at 25°C. GSH-Px activity was calculated based on the equation described by Flohe and Gunzier (31). SOD (EC 1.15.1.1) activity in the cytosolic fraction was determined as described by McCord and Fridovich (32) using the xanthine oxidase/cytochrome C system. The reaction mixture consisted of 2.4 mL of 0.05 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.3 mL of 0.1 mM ferricytochrome C, 0.1 mL of 1% deoxycholate, 0.1 mL of 1.5 mM potassium cyanide, 0.3 mL of 0.5 mM xanthine and 20 μL of appropriately diluted xanthine oxidase to produce a rate of reduction of ferricytochrome C at 550 nm of 0.025 absorbance unit/min at 25°C. One unit of SOD was defined as the amount of SOD required to inhibit the rate of reduction of cytochrome C by 50%.

Statistical analyses. The data shown are means ± SEM. Data were statistically analyzed using Student's *t*-test with Bonferroni adjustment (33), and $P < 0.05$ was considered significant.

RESULTS

Survivial, body weight and proteinuria levels. The body weights of B/W mice on all three diets showed a steady increase until four months, after which time the body weights plateaued (Table 2). The mice on the 10% CO diet showed slightly lower body weights after four months as compared to the mice on the KO or FO diets. The mice on 10% CO developed severe proteinuria relatively early (by seven months) compared to the mice on the n-3 lipid-based diets (Table 3). The three types of dietary lipids fed at the 10% level had a significant effect on the survival rates of these mice. The percent survival of B/W mice on the three dietary treatments is compiled in Table 4. At eleven months, all mice in the CO group had died, and 20% of the mice in the KO group and 47% of the mice in the FO group were still alive.

Fatty acid composition of microsomal fraction. In order to study the effect of dietary lipids on the membrane lipid composition of liver, microsomal membranes were isolated

TABLE 2

Effect of 10% (w/w) Corn Oil-, Krill Oil- or Fish Oil-Based Diets on Body Weights of (NZB×NZW) F_1 Mice

Age (months)	(body weights in grams)[a] Corn oil	Krill oil	Fish oil
1.0	20.2 ± 0.4	20.5 ± 0.4	20.0 ± 0.4
2.5	31.9 ± 0.7	33.7 ± 0.7	32.9 ± 0.5
4.5	40.8 ± 0.8	42.2 ± 1.0	41.8 ± 0.8
6.5	42.4 ± 1.6	46.1 ± 1.0	45.9 ± 0.9

[a]Values are means ± SEM of 20 mice/group.

TABLE 3

Effect of 10% (w/w) Corn Oil-, Krill Oil- or Fish Oil-Based Diets on Proteinuria Levels in (NZB×NZW) F_1 Mice

Age (months)	Diet	Number of mice	Proteinuria levels[a] + (30 mg/dL)	++ (100 mg/dL)	+++ (500 mg/dL)
6.5	Corn oil	15	9	1	5
6.5	Krill oil	15	8	4	3
6.5	Fish oil	15	11	4	0
10	Corn oil	5	0	0	5
10	Krill oil	11	3	1	7
10	Fish oil	14	5	2	7

[a]Proteinuria was determined by using Chemstrip (Boehringer Mannheim, Indianapolis, IN).

TABLE 4

Effect of 10% (w/w) Corn Oil-, Krill Oil- or Fish Oil-Based Diets on Survival Rate in (NZB×NZW) F_1 Mice

Diets	Survival (at 11 months)	
	Number of mice alive	Percent survival
Corn oil	0/15	0
Krill oil	3/15	20.0
Fish oil	7/15	46.7

[a]Both fish oil and krill oil significantly ($P < 0.01$) increased the survival rate of B/W mice.

and analyzed for fatty acid composition. KO and FO had generally similar effects on the fatty acid composition of the microsomal fractions, whereas the effect of CO was different (Table 5). The levels of 16:0 and 16:1 were significantly lower ($P < 0.001$), and the 18:0 level was higher in the CO-fed group compared to the KO- and FO-fed groups. The levels of 18:2 and 20:4n-6 fatty acids were significantly higher ($P < 0.001$) in the CO-fed group, and 20:5 and 22:6 were significantly higher ($P < 0.01$–0.001) in the n-3 lipids-fed groups. When comparing the KO and FO groups, we found that the levels of 18:1 ($P < 0.01$) were significantly lower and that the 22:6 levels were higher ($P < 0.01$) in the FO-fed group.

TBARS. To determine whether the different microsomal lipid composition affected the susceptibility of the microsomal lipids to peroxidation, we measured the generation of TBARS. We found that both the KO-fed and FO-fed groups showed significantly lower levels of TBARS values compared to the CO-fed group (Fig. 1).

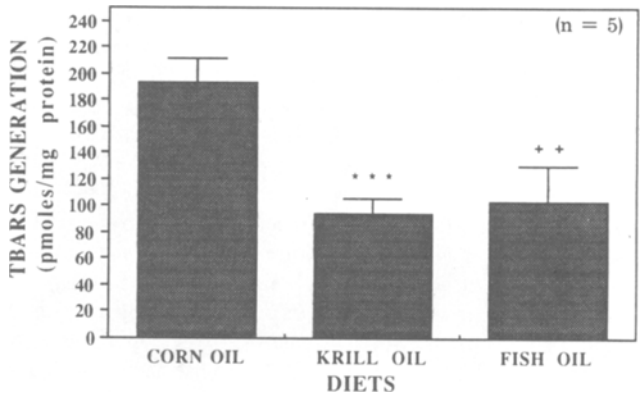

FIG. 1. Effect of 10% (w/w) corn oil-, krill oil- or fish oil-based dietary treatments on thiobarbituric acid reactive substances (TBARS) generation by liver microsomal membrane lipids of B/W mice. Values are means ± SEM of five mice/group. Values not denoted by the same superscripts are significantly different. TBARS generation was significantly higher in the corn oil-fed group compared to the groups fed fish oil ($^{++}P < 0.01$) or krill oil ($^{***}P < 0.001$).

mRNA Expression of antioxidant enzymes. The effect of the three dietary treatments on the expression of hepatic antioxidant enzyme mRNA patterns was examined by Northern blot analysis. Our data indicate that the three key antioxidant enzyme (CAT, GSH-Px and SOD) mRNA levels were lower in the livers of the CO-fed group com-

TABLE 5

Effect of 10% (w/w) Corn Oil, Krill Oil or Fish Oil on Hepatic Microsomal Fatty Acid Composition of B/W Mice[a]

Fatty acids	Corn oil	Fish oil	Krill oil
14:0	0.76 ± 0.04	0.98 ± 0.12	0.90 ± 0.20
14:1	0.49 ± 0.06	0.61 ± 0.11	0.64 ± 0.09
16:0	20.03 ± 0.49[b]	33.81 ± 0.43[c]	30.24 ± 1.03[c]
16:1	1.54 ± 0.30[b]	3.69 ± 0.38[c]	4.98 ± 0.45[c]
18:0	20.33 ± 5.11	13.96 ± 1.09	12.75 ± 1.34
18:1n-9	17.05 ± 1.86[b]	11.86 ± 0.89[c]	18.55 ±
18:2n-6	23.92 ± 1.24[b]	10.64 ± 0.53[c]	8.99 ± 0.31[c]
18:3n-3	0.36 ± 0.21	0.28 ± 0.05	0.17 ± 0.06
20:0	0.57 ± 0.52	0.15 ± 0.02	0.22 ± 0.03
20:1	0.30 ± 0.24	0.06 ± 0.04	0.14 ± 0.06
20:2	0.35 ± 0.12	0.12 ± 0.12	0.00 ± 0.00
20:3n-6	0.17 ± 0.10	0.00 ± 0.00	0.31 ± 0.18
20:4n-6	8.19 ± 1.37[b]	3.40 ± 0.08[c]	3.11 ± 0.43[c]
20:3n-3	0.15 ± 0.14	0.08 ± 0.08	0.19 ± 0.11
20:5n-3	0.00 ± 0.00[b]	7.04 ± 1.00[c]	8.59 ± 0.83[c]
22:0	0.35 ± 0.15	0.08 ± 0.05	0.00 ± 0.00
22:1	0.10 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
22:4n-6	1.08 ± 0.34	0.24 ± 0.09	0.17 ± 0.08
22:4n-3	0.00 ± 0.00	0.30 ± 0.09	0.40 ± 0.13
22:5n-6	0.88 ± 0.45	0.17 ± 0.10	0.07 ± 0.02
22:5n-3	0.00 ± 0.00[b]	0.78 ± 0.13[c]	0.61 ± 0.10[c]
22:6n-3	3.26 ± 0.32[b]	11.67 ± 0.50[d]	8.87 ± 0.63[c]
24:0	0.14 ± 0.06	0.11 ± 0.02	0.09 ± 0.02

[a]Values are expressed as percent w/w. Values are mean ± SEM of four mice/group. Abbreviation as in Table 4.
[b–d]Values with different superscripts in the same row are significantly different at $P < 0.05$ as revealed by Student's t-test with Bonferroni adjustment.

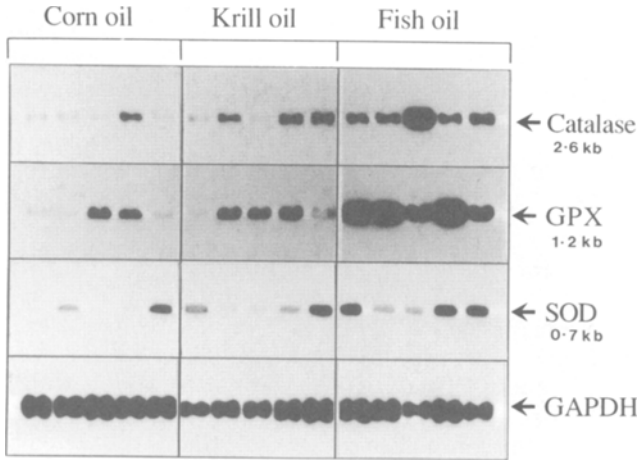

FIG. 2. Northern blot analysis of mRNA expression of antioxidant enzymes in the livers of B/W mice treated with 10% (w/w) corn oil-, krill oil- or fish oil-based diets. Each lane represents an RNA sample from one animal. Twenty micrograms of total RNA was denatured, electrophoresed through 0.8% agarose/formaldehyde gel, electroblotted on to nitrocellulase, and probed with random primed ^{32}P-labeled cDNA probes. The autoradiographic exposure time was eight days [catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD)] and 36 h for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

pared to those in the KO- and FO-fed groups (Fig. 2). FO feeding resulted in a higher expression of these enzymes compared to KO feeding. The differences in mRNA levels were not due to variation in RNA loading, as GAPDH levels did not vary significantly. Loading equivalency was also confirmed by ethidium bromide-stained 18S and 28S. Densitometric analysis of the autoradiographic bands, after standardization relative to GAPDH, revealed (Fig. 3) a significantly increased expression of the antioxidant enzymes in the FO group compared to the CO group ($P < 0.01$ level). However, when comparing the FO and the KO groups, CAT mRNA expression was significantly higher ($P < 0.05$) in the FO group.

Enzymatic activities of cytosolic CAT, GSH-Px and SOD. The mice fed the KO- and FO-based diets expressed significantly higher enzyme activities ($P < 0.001$) for CAT (9–9-fold), GSH-Px (4-fold and SOD (1.4-fold) in the liver cytosols when compared to the group given the CO-based diet (Table 6). The enzyme activities were similar in the KO and FO groups suggesting that n-3 lipids have a more favorable effect on antioxidant status than does CO.

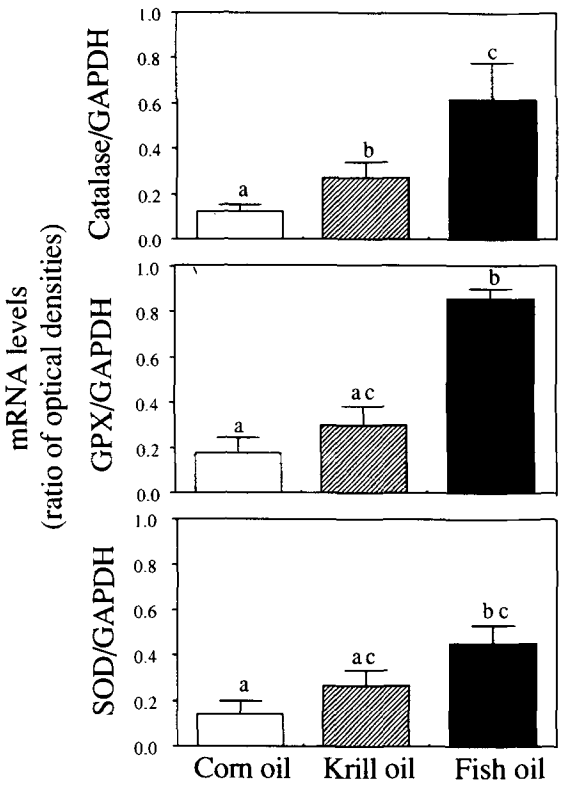

FIG. 3. Densitometric analysis of the autoradiographic bands seen in Figure 2. For the analysis, video image analysis was done utilizing the National Institutes of Health Image 1.4 program, and the results are expressed as a ratio of the specific message to the constitutively expressed GAPDH message. The values were tested for significance using Student's *t*-test. Any values in the figure not denoted by the same superscript are significantly different. The statistical significance of the values tested were as follows: CAT–CO vs. KO: $P < 0.05$; CO vs. FO: $P < 0.01$; KO vs. FO: $P < 0.05$; GSH-Px–CO vs. FO: $P < 0.01$; KO vs. FO: $P < 0.01$; SOD–CO vs. FO: $P < 0.01$. Abbreviations as in Figure 2; CAT, catalase; CO, corn oil; KO, krill oil; FO, fish oil; GSA-Px, glutathione peroxidase.

TABLE 6

Effect of 10% (w/w) Corn Oil-, Krill Oil- or Fish Oil-Based Diets on the Activities of Hepatic Cytosolic Antioxidant Enzymes in B/W Mice[a]

Enzyme	Corn oil	Krill oil	Fish oil
Catalase[b]	59.1 ± 4.4[c]	529.9 ± 27.5[d]	472.0 ±
GSH-Px[e]	25.5 ± 2.0[c]	98.7 ± 5.9[d]	106.0 ± 3.4[d]
SOD[f]	7.2 ± 0.3[c]	9.9 ± 0.4[d]	10.2 ± 0.2[d]

[a]Values are means ± SEM of five mice/group. Abbreviation as in Table 4.
[b]Catalase activity is expressed as μmol H_2O_2 reduced/mg protein/min.
[c,d]Values with different superscript letters (c,d) in the same row are significantly different at $P < 0.05$ level as analyzed by Student's *t*-test with Bonferroni adjustment.
[e]GSH-Px (glutathione peroxidase) activity expressed as μmol β-nicotinamide adenine dinucleotide phosphate oxidized/g protein/min.
[f]SOD (superoxide dismutase) activity is expressed in units/mg protein.

DISCUSSION

Along with several therapeutic approaches aimed at delaying the pathology of autoimmune diseases, nutritional intervention is now also being recognized as having a major effect in the treatment of several of these diseases (5,34–36). Our extensive work on dietary intervention in autoimmune mice suggests that both dietary restriction and an n-3 lipid diet administered without reducing the caloric intake can dramatically prolong the life span and delay the onset of immunological abnormalities in B/W mice (37–39). The exact mechanism(s) involved in delaying autoimmune disease either by food restriction or by FO, is presently not clear, although several possibilities have been suggested (4,5,9,34,35). It is well established that autoimmune diseases have multifactorial etiologies (1,12). Along with genetic, hormonal, viral, immunological and other factors, the inability of the antioxidant defense system to cope with oxidative stress has been proposed as one possible cause. More direct evidence, however, has not yet been forthcoming (39,40).

The role of antioxidant nutrients in membrane-related and cellular immune functions has been addressed by several investigators (41–44). Our earlier work on enzyme-dependent and nonenzymatic *in vitro* lipid peroxidation in B/W mice fed high levels of CO or FO has indicated that vitamin E supplementation can significantly reduce peroxidation in liver mitochondrial and microsomal membranes (45). We thus become interested in testing whether the degree of peroxidation in subcellular membrane lipids in autoimmune mice was due to defects in the antioxidant defense system. Furthermore, it would be important to establish whether different lipids have a differential effect on the antioxidant defense system at the molecular level. In the present study, we examined the effects of feeding n-6 and n-3 lipid-enriched diets on the activities and the mRNA expression of three key antioxidant enzymes, on microsomal lipid composition, and on the extent of lipid peroxidation. Our data show that the activities and the mRNA levels of these antioxidant enzymes were higher in the livers of B/W mice on the KO and FO diets, i.e., fed

lipids enriched with n-3 fatty acids. The degree of peroxidation and the levels of 20:4n-6 were also found to be significantly lower in the liver microsomal fractions of the n-3 lipids-fed B/W mice.

The effect of n-6 and n-3 lipids on subcellular membrane composition and function, including immune-related functions, has been reported by various investigators (46–48). Although several studies have pointed toward protective effects of FO, no information is available on the effectiveness of KO as an anti-inflammatory lipid. In recent years, consumption of krill has increased steadily in Japan and in some neighboring countries. Our present data indicate that crude KO may also have some anti-inflammatory potential, although insufficient information is available to compare its value to that of menhaden FO. The menhaden oil that was used in the present study was extensively purified and was odor-free, and it is currently used as n-3 lipid source in several experimental and clincial studies (5,34,49–51). As the three dietary lipids we used in the present study were supplemented with equal levels of antioxidants, the results we obtained could not have been compromised because of differences in dietary antioxidant levels.

Recent studies have clearly established that the source of nutrients and the caloric intake can both play an important role in determining the expression of certain genes (19,52,53) and in determining the cellular antioxidative defense mechanism (15,43,54,59,60). As the antioxidant enzymes GSH-Px and CAT detoxify hydroperoxides and SOD detoxifies superoxide radicals, they all play along with antioxidant nutrients, a key role in the antioxidant defense system. The antioxidant enzymes constitute a major cell defense against acute oxygen toxicity, and they protect membrane components against damage caused by free radicals. Nutritional intervention, including food restriction, preserves the enzyme activities and the mRNA expression of these antioxidant enzymes, as well as cytochrome P450 mRNA levels (10,38,57,59,60). Both the quantity of the dietary fat and its composition affect the hepatic H_2O_2-metabolizing systems, the activities of NADPH-generating enzymes and lipid peroxidation (58). Interestingly, both CAT and GSH-Px activities were found to increase when polyunsaturated fatty acids in the diet were increased (20). Yamazaki *et al.* (61) found that feeding FO to animals increased the activities of peroxisomal enzymes, fatty acyl-CoA, CAT and GSH-Px and also lowered plasma triglycerides when compared to safflower oil-fed animals. The results of the present study strongly suggest that the mRNA levels of the three key enzymes involved in the antioxidant defense are lower in the livers of CO-fed B/W mice (at 6.5 months of age) than in the livers of FO- or KO-fed mice. The findings suggest that the defect may be at the transcriptional level. Decreased antioxidant mRNA levels were observed with progressive renal disease in these mice. It is not clear at present at what time point the antioxidant enzyme mRNA levels begin to decline in CO-fed mice compared to FO-fed mice, and this still needs to be established. Additional studies are required to clarify the exact role of specific lipids and the levels that would affect antioxidant enzyme mRNA levels both in autoimmune disease-prone strains and in resistant strains. Increased levels of antioxidant enzymes, including SOD and CAT as well as of other antioxidants are closely linked to preserving health and longevity in both mice and rats (10,14,57).

Reactive oxygen species have also been implicated as possible effectors of oxygen-mediated changes in gene expression (61). Although intracellular CAT is thought to protect macrophages from the toxic effects of high doses of H_2O_2, GSH-Px is thought to be more important than CAT in hydroperoxide catabolism (63). It is quite possible that certain abnormal immune functions in CO-fed B/W mice could be caused by increased peroxidative damage that occurs in cellular membranes due to changes in the activities of antioxidant enzymes causing increased oxidative stress to cells.

A very interesting observation made in the present study is the significant difference in steady-state mRNA levels for the key antioxidant enzymes found in the livers of mice on two different lipid diets. Recent studies have suggested that n-6 fatty acids are more susceptible to lipid peroxidation than are n-3 fatty acids. Fatty acids of the n-6 type appear to be oxidized rapidly under conditions under which n-3 fatty acids were not oxidized at any detectable rate (64,65). Although the type of dietary lipid and the degree of unsaturation of membrane phospholipids have a strong effect on lipid peroxidation (66), additional factors, including free radical chain breaking antioxidants such as vitamin E, initiators of lipid peroxidation such as mixed function oxidase system, and inherent antioxidant defense system (16–18), may also be involved.

Earlier studies on MRL/*lpr* mice have indicated that, in addition to immunological abnormalities, these mice exhibited altered membrane phospholipid fatty acid composition in subcellular fractions, including in liver nuclear envelopes and liver mitochondria (67). Also, the genotype had a definite effect on splenocyte phospholipid fatty acid composition (68), suggesting that higher levels of 20:4n-6 and of some longer chain fatty acids could make certain membranes more susceptible to peroxidation, for example, by decreasing fluidity and thereby affecting the activity and expression of enzymes (73). Although hepatic antioxidant enzyme expression and activities and microsomal fatty acid compositions of mice were similar in the KO and FO groups, the survival rate was higher in the FO group, suggesting FO may be more effective in preventing the onset of autoimmune disease by acting more effectively on target tissues, such as kidneys, and by modulating other immune factors (69,70).

In the present study, the three dietary oils used were supplemented with 2.5 g vitamin E/kg of oil to prevent peroxidation, as recommended by the NIH. Each diet therefore contained ~215 mg vitamin E/kg (vitamin E from oil and vitamin mixture). Although the vitamin E levels in plasma or hepatic microsomes were not determined in the present study, this issue had previously been addressed by us. For instance, we had compared the effects of 75 and 500 I.U. of vitamin E in FO- and CO-based diets on the vitamin E levels in plasma and hepatic microsomal and mitochondrial membranes and on lipid peroxidation. We found that in FO-fed mice, 75 I.U. of vitamin E is inadequate to restore the loss of plasma and membrane-

bound vitamin E, and 500 I.U. was sufficient to replenish both serum and hepatic vitamin E levels and to reduce free radical generation (45).

Several studies have indicated that tissue lipid peroxidation may result from lowered antioxidant enzyme activities (19,71) as well as from low tissue vitamin E levels (18,72). However, by increasing dietary vitamin E levels, peroxidation of hepatic microsomes could be prevented (18,72). Also, by increasing dietary vitamin E levels from 35 to 180 I.U., increased tissue vitamin E levels in the FO group similar to those in the CO group were noted, whereas liver and kidney vitamin E levels were unaffected by dietary lipids (18). Although hepatic vitamin E levels were not measured in the present study, our earlier observations (45) and studies reported by others suggest that tissue vitamin E levels could be increased with an increase in dietary vitamin E levels. It is possible, however, that the high level of dietary vitamin E used in the present study may have protected the target tissues of autoimmune-prone B/W mice from peroxidative damage, especially in FO-fed mice. In addition, the high dietary vitamin E supplementation and the well-designed feeding and storage practices followed for oils in the present study may have contributed further to preserving the enzyme activities and the mRNA expression of enzymes involved in the antioxidant defense system far better in FO- and less so in CO-fed mice. Further work is required to measure the effects with and without different levels of vitamin E on tissue vitamin E levels and lipid peroxidation as well as on antioxidant enzyme activities.

In summary, our data indicate that a diet containing marine lipids with very long-chain n-3 fatty acids may delay the onset of autoimmune disease in mice. It appears that alteration of the membrane fatty acid composition due to n-3 lipids may enhance the efficiency of the antioxidant defense system at the molecular level and may prevent the susceptibility of membranes to peroxidation. Further studies are needed to establish the role of various lipids in maintaining an antioxidant defense system and in assuring an extended life span in long-lived inbred strains of mice and rats. As FO contains highly unsaturated and potentially unstable n-3 fatty acids, concerns have been raised in the past about their tendency to peroxidize during storage, as animals fed FO have been reported to excrete higher levels of TBARS in urine (74). Our data, however, would indicate that by supplementing FO with adequate levels of antioxidants, it is possible to enhance the antioxidant defense system, especially in autoimmune-prone mice. This may be one of the mechanisms by which the FO diet significantly increases the survival rate and delays the onset of autoimmunity in autoimmune prone B/W mice. The findings are encouraging in view of utilizing FO in therapeutic applications.

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Serum Fatty Acid Profiles in Cystic Fibrosis Patients and Their Parents

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Fatty acid compositions of the major serum lipid classes from 43 cystic fibrosis (CF) homozygotes (CF patients), 36 obligate heterozygotes (parents of CF patients) and 34 controls were determined by capillary gas chromatography. Fatty acid compositions of the homozygote CF patients were skewed in the direction of relative essential fatty acid deficiency in comparison with the controls. Less pronounced, but similar deviations from normal, were observed in the heterozygotes. Homozygotes with normal fatty acid compositions and heterozygotes with considerably disturbed fatty acid profiles were found.
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In several studies of the fatty acid (FA) composition of serum lipid classes in cystic fibrosis (CF) patients (cited in Ref. 1), CF seems associated with some form of essential fatty acid (EFA) deficiency. Studies of FA in CF patients have been related to age (1,2), presence of pancreatic insufficiency (1,3) or degree of fat malabsorption (1). Polyunsaturated fatty acid (PUFA) profiles of a series of CF patients previously studied in this laboratory revealed distortions of PUFA pattern in serum lipids, indicating linoleic acid deficiency and probable abnormalities of PUFA metabolism (4).

FA compositions of the serum lipids of small groups of obligate heterozygotes (parents of CF patients) have been reported (3,5). Because biochemical differences or abnormalities may occur in heterozygotes due, among other causes, to the presence of certain mutations of the CF gene, a large group of heterozygotes was investigated in comparison with homozygotes and healthy controls, using capillary gas chromatography, which is more discriminating and sensitive than the packed columns used in earlier studies.

Lower linoleic acid (18:2ω6) in serum phospholipids (PL) of CF patients has been found in most studies, but subdivision of CF patients on the basis of this measure had not been done. We chose this criterion for study of CF patients and of obligate heterozygotes because it should reveal changes in other FA in relation to linoleate level, and reveal whether these relationships were dissimilar in CF homozygotes, CF heterozygotes, and healthy controls.

MATERIALS AND METHODS

Serum. Blood was obtained in the morning from nonfasting individuals who gave informed consent. Sera were frozen at –30°C and analyzed within a week. This study was approved by the Human Subjects Committee of the University of Minnesota (Austin, MN).

CF homozygotes. CF patients (24 males and 19 females, ranging in age from 4 to 44 years with a mean age of 20.2 years) were outpatients who visited the University of Minnesota Cystic Fibrosis Center. Diagnosis was based on clinical criteria and multiple sweat tests showing chloride concentrations greater than 60 mmol/L with the Gibson–Cooke sweat test (6). Their weight for height ranged from 70.4 to 128.1% of the predicted value (mean ± SD = 101.3 ± 13), their forced vital capacity from 33.1 to 117.6% (average 85.0 ± 23.6) and forced expiratory volume from 16.8 to 121.4% (average 69.2 ± 29.9). The CF patients were instructed to consume normal diets and to adjust their pancreatic enzyme therapy to have one or two bowel movements a day. All were encouraged to increase their dietary fat intake, but none were on medium chain triglycerides (TG). (See Table 1 later for details.)

Heterozygotes. Twenty-three mothers and 14 fathers of CF patients volunteered for this study. They ranged in age from 25 to 59 years of age (with a mean age of 38.7 years; all asymptomatic and in good health).

Controls. This group consisted of 17 males and 17 females, all healthy and without a family history of CF, with an average age of 33.6 years and a range of 21–45 years. This group included healthy parents of children with other diseases, as well as volunteers from the University of Minnesota staff.

Assay. Serum lipids were extracted and separated into PL, cholesteryl esters (CE), TG and nonesterified fatty acids (NEFA). From each lipid class, methyl esters were prepared in methanol by boron trifluoride catalysis (7). Methyl esters were separated and analyzed by gas chromatography using a 15 m × 0.1 mm i.d. CPS 1 capillary column (Scientific Glass Engineering, Inc., Austin, TX), in a Packard (Palo Alto, CA) 428 gas chromatograph equipped with a glass splitter and a flame-ionization detector. The split ratio was 1:100, the column flow 0.11 mL/min and, after an initial delay of 10 min at 160°C, the temperature was programmed from 160 to 200°C at 2°C/min and held at 200°C for 35 min to complete an analysis in 65 min. Identification of the methyl esters was made by comparing their retention times with those of authentic standards.

Data handling. Content of an FA is expressed as percent of total FA of a lipid class, which is most appropriate for expressing concentration of an FA within a lipid substrate (8–12). Data were entered into a computer by group and lipid class. The content of linoleic acid in the serum PL (18:2ω6) was used to assign each individual into subgroups with low (<15%), intermediate (15–20%) and "normal" linoleic acid content (>20%). Programs, written to

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Abbreviations: CE, cholesteryl esters; CF, cystic fibrosis; DBI, double bond index; EFA, essential fatty acid(s); FA, fatty acid(s); NEFA, nonesterified fatty acid; NR, normalcy ratio(s); PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglycerides.

calculate and plot profiles of normalcy ratios (NR) (equivalent to Z scores) to evaluate EFA status of one group compared to its control group (9,10), were modified to present profiles of homozygotes and heterozygotes together. Significance of differences between two groups was evaluated by Student's *t*-test. One-way analysis of variance was used for comparisons of the three subgroups differing in PL-18:2ω6.

RESULTS

The characteristics of the three groups and their three subgroups are listed in Table 1. Only one individual (3%) in the control group (n = 34) had less than 20% linoleic acid in her serum PL. This occurred for 46% of the parents of CF patients (n = 37) (43% in males, n = 14; and 48% in females, n = 23) and for 70% of the CF patients (n = 43) (67% in males, n = 24; and 74% in females, n = 19). Thus, 30% of the patients and 54% of the carriers of the CF gene had normal serum levels of linoleic acid. Table 1 also shows that a few heterozygotes had lower PL-18:2ω6 than some CF patients.

In contrast to control individuals with no known metabolic disease (13), the percentage of 18:2ω6 in the PL of CF patients appeared to increase with age. This was confirmed by linear regression analysis of 18:2ω6 in PL vs. age, for which the equation was found to be:

$$\text{PL-18:2}\omega6 = 15.34 + 0.13\,(\text{yr}) \quad R = 0.35\ (0.01 < P < 0.05) \qquad [1]$$

No correlation was found between PL-18:2ω6 and the relative weight for height of the patients.

The FA composition of serum PL of the control group ± SE is given in Table 2. Values for PL for CF patients and parents of CF patients may be estimated from these control values and the NR shown in Figure 1.

FA profiles in CF. Although some FA and parameters calculated from them have been used as measures of EFA status, we consider that the whole profile is more revealing. It graphically depicts the FA composition of a tissue lipid by comparing it with control values, and permits differentiation of many PUFA defects occurring in disease. Profiles of serum PL and NEFA of CF homozygotes and heterozygotes are given in Figures 1 and 2.

The FA profile of PL in CF patients (Fig. 1, upper bars of each pair) showed low 18:2ω6 and 20:0 in serum PL, and high 16:1ω7, 18:1ω9, 20:3ω6, 22:5ω3 and 24:1ω9. The indicator of nutritional EFA deficiency, 20:3ω9, did not differ significantly from normal value. Loss of 18:2ω6 was largely replaced by monoenoic acids and minor significant increments of 20:3ω6 and 22:5ω3. Total ω6 PUFA were significantly suppressed in homozygotes, whereas total ω3 PUFA were normal. Despite these changes, a composite indicator of lipid "fluidity," the double bond index (DBI) of the FA of PL (8), did not vary significantly from normal (1.36 ± 0.03 vs. 1.37 ± 0.03).

Parents of CF patients (Fig. 1, lower bars of each pair) showed most of these changes but with less significance. Thus, 18:2ω6 was significantly less than normal ($P < 0.001$), although it was not as low as for homozygotes. Although the heterozygote profile had few significant abnormalities or dramatic features, the contour was similar to that of homozygotes. The DBI of the FA of PL was slightly but significantly less in the heterozygotes than in the controls.

The CE profile for CF patients revealed low 18:2ω6 but elevated 18:3ω6 and 20:3ω6. The major compensation for the low 18:2ω6 was by 18:1, 16:0 and 16:1. No 20:3ω9 could be detected in this lipid class. DBI was decreased (1.55 ± 0.02 vs. 1.68 ± 0.001; $P < 0.001$), and branched-chain and odd-chain acids increased. Parents of CF patients had less 18:2ω6 and elevated levels of 18:1 and saturated acids. Data for CE are not shown.

The TG profile (data not shown) for CF patients had low 18:2ω6. Paucities of 18:3ω3 and its end product, 22:6ω3, occurred in this lipid class only, perhaps reflecting deficient intake of ω3 PUFA. Increased 20:3ω9, usually taken as evidence of EFA deficiency, occurred only in this lipid.

TABLE 1

Description of the CF Patients, Parents of CF Patients and Control Groups and Their Subgroups Studied[a]

Groups/subgroups	n	%	Male	Female	Mean	SD	Age range (yr)
CF patients	43	100	24	19	20.2	8.5	4–44
Low <15% PL-18:2ω6	8	19	4	4	16.6	5.7	11–24
Int. 15–20% PL-18:2ω6	22	51	12	10	18.7	8.5	4–31
Norm. >20% PL-18:2ω6	13	30	8	5	24.7	8.5	13–44
Parents of CF Patients	37	100	14	23	38.7	9.4	25–59
Low	5	14	3	2	41.2	10.4	33–59
Int.	12	32	3	9	36.6	9.0	29–59
Norm.	20	54	8	12	39.2	9.7	25–53
Controls	34	100	17	17	33.6	7.2	21–45
Low	0	0	0	0	—	—	—
Int.	1	3	0	1			
Norm.	33	97	17	16	33.8	7.0	21–45

[a]CF, cystic fibrosis; PL, phospholipids; Int., intermediate group; Norm., normal group.

TABLE 2

Fatty Acid Composition[a] ± SEM of Serum Phospholipids of the Control Group

FA	%	FA	%	FA	%
14:0	0.36 ± 0.02	18:1ω9	8.59 ± 0.22	20:4ω6	10.1 ± 0.27
14:1ω5	0.10 ± 0.02	18:2ω6	23.6 ± 0.47	22:0	0.90 ± 0.06
15:0	0.40 ± 0.09	18:3ω3	0.16 ± 0.03	22:4ω6	0.53 ± 0.03
16:0	24.3 ± 0.35	18:3ω6	0.19 ± 0.02	22:5ω3	0.77 ± 0.05
16:1ω7	0.29 ± 0.06	20:0	0.37 ± 0.8	22:5ω6	0.88 ± 0.06
17:0	0.45 ± 0.01	20:1ω0	0.13 ± 0.03	22:6ω3	2.53 ± 0.15
18:0Br	0.25 ± 0.03	20:2ω9	0.24 ± 0.05	24:0	0.84 ± 0.07
18:0	12.1 ± 0.24	20:3ω6	2.98 ± 0.11	24:1ω9	0.77 ± 0.06
18:1i	1.68 ± 0.17	20:3ω9	0.46 ± 0.05		

[a]Weight %. Branched isomers are identified by Br, positional monoenoic acid isomers different from ω9 by i. FA, fatty acids.

Branched-chain acids were elevated 5.8-fold, but odd-chain acids were normal. The 16:1 was elevated, total PUFA were low and the DBI was 88% of normal. Parents of CF patients presented a pattern similar to CF patients, with diminished magnitudes and significances.

The NEFA profile for CF patients (Fig. 2, upper bars) showed low 18:2ω6, total ω6, PUFA and 20:0, as well as significantly elevated 20:3ω6, 16:0, 18:0, and odd-chain FA. The profile for parents of CF patients was similar (Fig. 2, lower bars), with lesser changes and significances. The unique feature of these profiles was that in both homozygotes and heterozygotes, 20:3ω6 was elevated to four times the normal, suggesting that the eicosanoid pattern may be skewed, affecting metabolic control.

Studies comparing subgroups. With decreasing proportion of 18:2ω6 in the PL of the three subgroups of CF patients, there was a decrease of this FA in CE, TG and NEFA as well. A regression analysis between PL-18:2ω6 and CE-18:2ω6 was made, and the result is shown graphically in Figure 3.

When the subgroup of CF patients with highest normal PL-18:2ω6 content was compared with controls, those differences characteristic of CF were much less pronounced (data not shown). Eleven patients with normal values for PL-18:2ω6 could be matched with 11 individuals from the control group having very similar PL-18:2ω6 values. There were no significant differences with respect to other FA when comparing these two groups. That is, patients

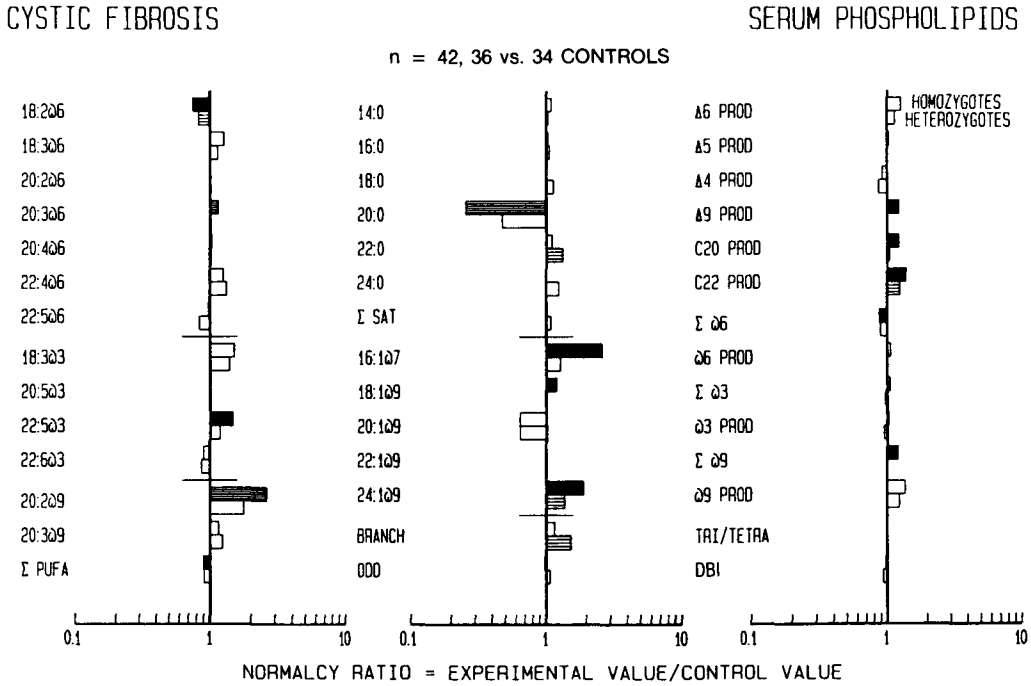

FIG. 1. Fatty acid profile of serum phospholipids from cystic fibrosis (CF) patients (top bars) and parents of CF patients (lower bars) as compared with controls. Normalcy ratio, the experimental value divided by control value, is plotted on a logarithmic scale. Black bars $P < 0.001$, closely-striated $P < 0.01$, widely-striated $P < 0.05$, open bars nonsignificant.

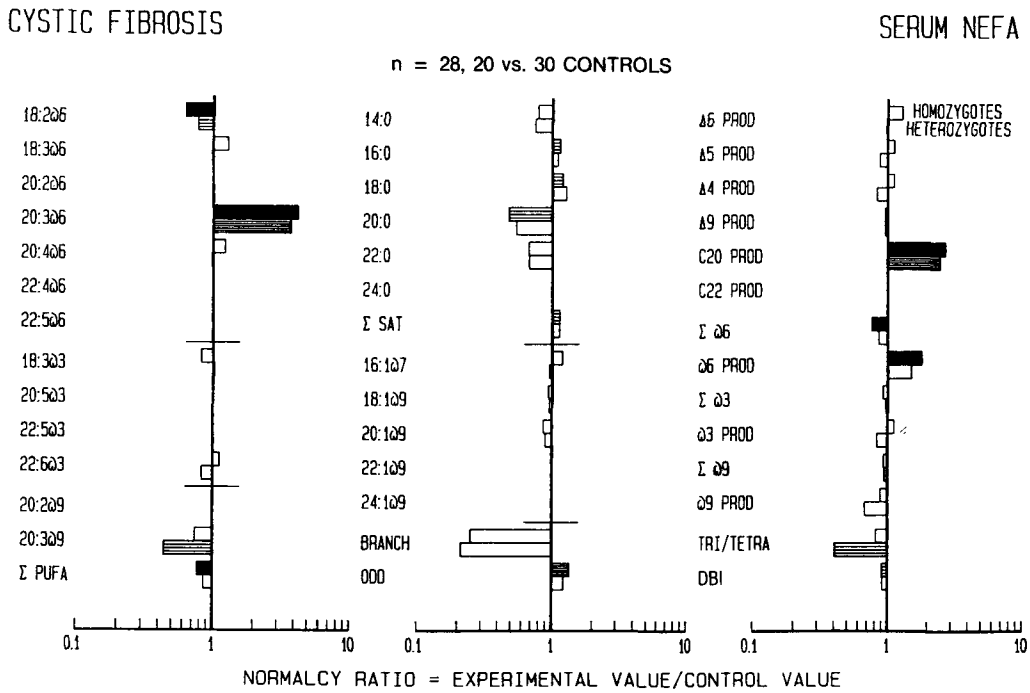

FIG. 2. Fatty acid profiles of fatty acids of serum nonesterified fatty acids from CF patients (upper bars) and from parents of CF patients (lower bars). See Figure 1 for legends and abbreviations.

who were normal in 18:2ω6 were normal in all other FA in the PL fraction. With stepwise decreases in 18:2ω6 in the homozygote subgroups, 14:0, 15:0, 16:0, 16:1ω7, 18:1ω9, 18:3ω3, 20:2ω9 and 24:1 increased, whereas 17:0, 18:0Br, 20:0 and 20:1ω9 decreased. In going from the subgroup with normal 18:2ω6 to the intermediate group, increases in 18:3ω6, 20:3ω9, 20:3ω6, 20:4ω6, 22:5ω6 and 22:6ω3 were observed, whereas with further decrease in 18:2ω6, these decreased. Reversed nonlinear changes were observed for 16:1*t*, 22:0 and 24:0. All the fatty acids of the former group have in common a Δ6 desaturase step in their metabolism.

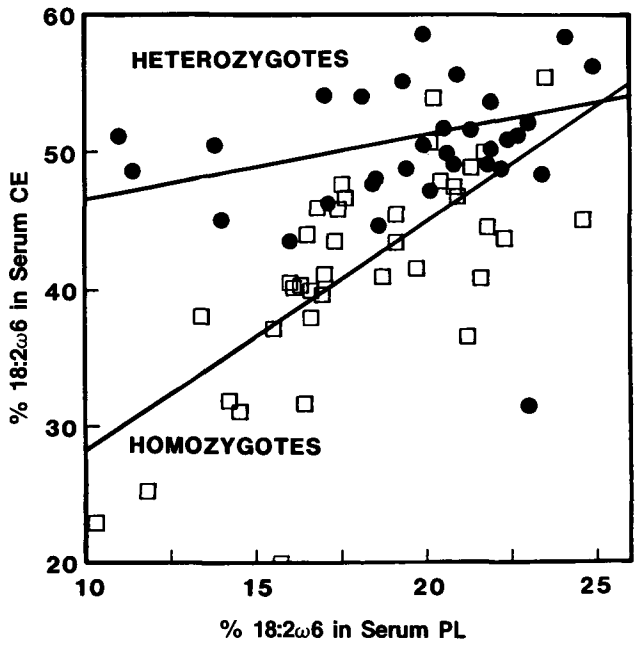

FIG. 3. Correlation between the content of linoleic acid in serum phospholipids and serum cholesteryl esters (CE) in cystic fibrosis (CF) patients (□—□) and in parents of CF patients (●—●).

With decreasing proportion of 18:2ω6 in the CE of the three subgroups of CF patients, there was an increase in 16:0, 16:1ω7, 18:1ω9 and 18:1 isomers. In TG, the 16:0 and 16:1ω7 increased as the 18:2ω6 decreased. In the NEFA fraction, 16:1ω7 increased only in the subgroup with severely reduced 18:2ω6 content.

When the homozygote and heterozygote subgroups with normal PL-18:2ω6 were compared, no significant differences were found for any FA or calculated parameter. When the subgroups with subnormal PL-18:2ω6 were compared, the homozygotes with low PL-18:2ω6 had a deficit in 18:2ω6 of about 11% of the total FA in this lipid class, largely compensated for by a 7% increase in monoenoic acids and about a 1.5% increase in saturated FA. In contrast, in the heterozygotes with low PL-18:2ω6, the 11% deficit of 18:2ω6 in the PL was compensated for by an equal increase in saturated FA, but not by a change in monoenoic acids. A regression analysis between oleic acid and linoleic acid in serum PL of CF patients of CF heterozygotes is shown in Figure 4. In these low PL-18:2ω6 subgroups, the lowered 18:2ω6 in the CE was compensated for by an increase in saturated FA and by an increase in monoenoic acid which was more pronounced in the homozygote subgroup than in the heterozygote subgroup. In the TG, only 18:2ω6 was significantly different in the low PL-18:2ω6 subgroups. There was no significant difference in composition of the NEFA between the homo-

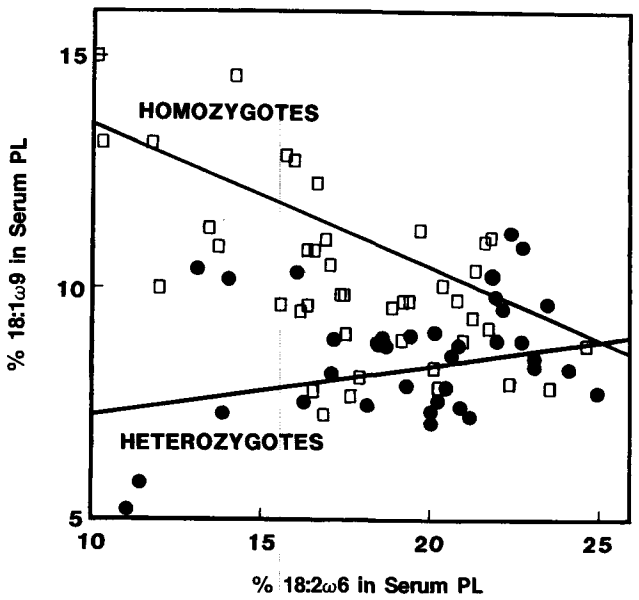

FIG. 4. Correlation between the content of oleic and of linoleic acids in serum phospholipids (PL) in cystic fibrosis (CF) patients (□—□) and in parents of CF patients (●—●).

zygote and heterozygote subgroups with low PL-18:2ω6. These observations indicate that homozygote and heterozygote conditions modify the acylation-deacylation reactions differently.

DISCUSSION

Methodology. It was not feasible to obtain blood in the fasting state because most patients and their accompanying parent(s) were required to travel a considerable distance to reach the clinic. The FA composition of PL and CE are not influenced in the postprandial state (14). The FA composition of the TG and NEFA, on the contrary, change toward that of the fat fed before returning to fasting values in a time- and dose-dependent matter (14). Nevertheless, valuable information can be obtained from these lipid classes. Indeed, an increase in 20:3ω6, as seen in the NEFA fraction, cannot be due to fat consumption because neither this FA nor its precursor, 18:3ω6, is present in a Western type diet to any significant extent (15), and fat feeding would tend to reduce its level in these lipids.

"Two flag" profiles, as introduced here, are extremely useful to visualize similarities and dissimilarities of deviations of two groups from the norm. FA profiles, especially of the PL, indicate EFA status of a group. We subdivided the entire group of patients and CF-heterozygotes into three subgroups. The lower limit of the group with "normal" PL-18:2ω6 was chosen as 20% PL-18:2ω6 because all control individuals except one (who was 2.6 SD lower than the mean and may have been an unknown carrier, *vide infra*) fell in this group. The upper limit of the "low-linoleic acid group" was chosen as 15% PL-18:2ω6. When subclassification was done in this way, the average of PL-18:2ω6 of the total group was almost identical to that of the intermediate subgroup.

CF patients. The 18:2ω6 in PL is reduced in CF (1–4, 16,17). When subgroups with decreasing proportions of PL-18:2ω6 were examined, they also exhibited decreasing proportions of 18:2ω6 in CE, TG and NEFA. Thus, the four lipid classes reveal rather parallel phenomena. Ninety-seven percent of those in the normal group had PL-18:2ω6 > 20% (Table 1). The only control person of 34 to have low PL-18:2ω6 may be an unknown CF heterozygote. Indeed, the carrier frequency is about 1:20 in a Caucasian population (18), and reduced PL-18:2ω6 occurs in 47% of the carriers (this study). In contrast, 70% of the CF patients had less than this proportion of 18:2ω6 in their PL. This proportion is close to the 85% of CF patients with malabsorption found to have reduced levels of 18:2ω6 in total plasma lipids (1). The increase in PL-18:2ω6 with age observed here in the CF patients is in contrast with the very slight decrease with age observed in control individuals (13), suggesting that a selection among the patients may have taken place, permitting a higher survival of those with a higher, more normal PL-18:2ω6 content. The basis of this hypothetical selection could possibly be that this group may contain more patients with pancreatic sufficiency. Indeed, normal levels of 18:2ω6 in PL and better survival rates have been found in subgroups of patients without pancreatic insufficiency (3) and without malabsorption (1), but not in patients with fat malabsorption corrected by administration of lipases (2,19). Much larger groups and prospective studies will be required to decide whether 18:2ω6 of PL is a good predictor of survival in CF patients. Higher PL-18:2ω6 may be the reflection of better nutritional status (20). Nutritional rehabilitation in CF has been shown to result in a catch-up weight, sustained improvement in linear growth and fewer pulmonary infections, as well as a significant reversal of the trend for deteriorating lung function (21). On the other hand, interventions aimed at correcting linoleic acid deficiency in CF without change in caloric intake also resulted in enhanced growth (22). Farrell *et al.* (1) found no correlation between age and content of 18:2ω6 in plasma total lipids of CF patients. Their observation is not necessarily at variance with our finding because changes in the content of 18:2ω6 in nonfasting total lipids do not necessarily parallel changes of 18:2ω6 in PL. Improvement of linoleic acid status after administration of extra calories as carbohydrates (20) or after administration of linoleic acid-rich diets (16,21,22) has been documented. Monoene acids are increased in the PL of CF patients (1,4,20). Some authors (4,23) found decreased levels of 20:4ω6 in the PL fraction of patients with CF, whereas others did not (1–3). Whether 20:4ω6 is normal may depend on several factors including the plasma zinc level (24) and the extent of fat malabsorption (19) and liver involvement (23). Normal (4,25), as well as significantly increased (26), levels of 20:3ω9 have been described in PL of CF as well as slightly decreased (4,25) to increased levels of 20:3ω6 (26,27). Our present findings suggest that when 18:2ω6 is slightly low, 20:3ω6 and 20:4ω6 may increase in compensation and that when 18:2ω6 is reduced more markedly, synthesis and incorporation of its metabolites may be subnormal. Lower levels of 18:2ω6 than found in this study group may be required to get an increase in 20:3ω9, as is the case in

CF infants (17). CF infants were not included in this study. An increase of PL-20:4ω6 with less severely reduced PL-18:2ω6, and a decrease of 20:4ω6 with sharply reduced 18:2ω6 was described in a patient with fat malabsorption maintained by intravenous alimentation preparations with different 18:2ω6 contents (28). The change of 20:4ω6 with decreasing 18:2ω6 seems related not to CF but to the degree of EFA deficiency.

NEFA, although the least abundant of the four lipid classes, vary with prandial state, have the highest rate of turnover and include many eicosanoid precursors important to metabolic control. This lipid class changes drastically in some diseases, with major changes of pattern and concentration of PUFA. In Reye's Syndrome, NEFA are elevated, and the proportions of PUFA may reach ten times normal values (11). In cirrhosis, 20:2ω6, 20:4ω6 and 22:6ω3 increase 2–3-fold (12). These observations suggest release of PUFA from PL to NEFA.

The deviations from normal composition of TG, CE and NEFA in CF are similar to those reported by others (2–5), and similar, but more severe, deviations have been reported for patients with EFA deficiency (10,28–30). The content of 18:2ω6 in the lipids of CF patients is not as low as in severe EFA deficiency. In the latter condition, there are high levels of 20:3ω9. A marked increase of 20:3ω9 was not found in this study, thus not indicating defective EFA metabolism as reported earlier (4,31). Our present study indicates a moderate deficiency of 18:2ω6 for the group as a whole. The difference between our earlier study (4) and the present one may be due to improved management of the disease over the years.

The FA profile of PL in CF was found to be different in the different subgroups (results not shown). Thus, the profile of the total group will critically depend upon the abundance of the different subgroups or more generally on the percentage of 18:2ω6 distribution of the total group. This implies that in comparisons of FA profiles in different diseases and attempted correlations of other FA with 18:2ω6, the distributions of 18:2ω6 should be the same in the compared groups. Moreover, comparison of FA profiles in different diseases may be complicated by minor differences due to age and sex distribution (13), and to larger differences due to differences in eating habits.

With these reservations in mind, we compared some features of the FA profile of the CF subgroup with intermediate linoleic acid content with those of some other groups with a similar NR for linoleic acid. In chronic malnutrition, which is generally believed to be largely caloric, and protein deficiencies (32), reduced PL-18:2ω6 (NR = 0.79) was accompanied with significantly ($P < 0.001$) reduced 20:3ω6 (NR + 0.64) and 20:4ω6 (NR = 0.72). In anorexia nervosa (33), where energy intake is reduced but protein intake is normal, reduced PL:18:2ω6 (NR = 0.78) was accompanied with essentially normal 20:3ω6 (1.03) and 20:4ω6 (NR = 0.95). In healthy individuals put on a diet with 0.6 to 1.3 energy % linoleic acid (34), reduced PL-18:2ω6 (NR = 0.73) was associated with increased 20:3ω6 (NR = 1.46) and 20:4ω6 (NR = 1.20). In the CF subgroup with identically reduced PL-18:2ω6 (NR = 0.73), there was significant increase ($P < 0.001$) in 20:3ω6 (NR = 1.09). Thus, deviations in the abundance of the major ω6 FA in serum PL of this CF group most resemble those seen in those in the normal group with low-linoleic acid intake. Several factors, such as incomplete correction of fat malabsorption, reduced energy intake, increased arachidonic acid turnover (35) and increased energy expenditure (36), may increase linoleic acid requirement in CF. Thus, suboptimal intake of linoleic acid could explain these results.

Parents of CF patients. An early study found no differences between the FA compositions of the major serum lipid classes of those in the normal group and of five obligate CF heterozygote (3). We found the same in our subgroup of heterozygotes with normal 18:2ω6 in PL (results not shown) who comprised 54% of our heterozygotes (Table 1). We also found heterozygotes with subnormal 18:2ω6 in PL in whom abnormal FA compositions of the other serum lipids were observed. In a study on the NEFA pattern of CF patients and their parents, opposing deviations from normal were found for 16:0 and 18:2ω6 (5). The authors suggested that increased 18:2ω6 in parents of CF, observed in that study, might be due to a higher dietary intake of PUFA (5). Differences in lipoprotein composition between non-CF siblings and those in the normal group have been described (37). Comparisons between homozygote and heterozygote subgroups with similar PL-18:2ω6 content indicate that changes in compensation for the paucities of EFA differ for homozygotes and heterozygotes.

Reduced content of linoleate in serum lipids in CF patients may be secondary to malabsorption (1), but fat malabsorption has not been found in heterozygotes. Our finding that some heterozygotes have reduced 18:2ω6 content in their serum lipids could suggest that at least in some CF gene carriers, the reduced 18:2ω6 levels have other causes than fat malabsorption. Specific mutations are associated with increased energy expenditure (36) which, in turn, could affect linoleate levels. Heterozygotes may differ from the control group because parents may adapt their diet to that of their sick child. Because our CF patients were instructed to eat normal diets, this possibility seems unlikely. Heterozygotes with severely disturbed FA compositions have no clinical signs of the disease, whereas homozygotes with normal FA composition display clinical signs of the disease. Disturbed FA compositions do not seem to be strongly related to the clinical symptoms.

This does not mean that normalizing FA compositions of plasma lipids in CF (16,20,22,38) is not beneficial, although improvement in clinical status of the patients in response to linoleate intake has not been uniform (1,4,39,40). In our present study, we found no evidence for the deficiencies of long-chain PUFA observed ten years previously (4), and we suspect that generally improved care and nutrition may have exerted an influence. From the regression lines in Figure 4, it is apparent that differences found between CF homozygotes and heterozygotes are greatest at low levels of PL-18:2ω6, and that they vanish for individuals with normal PL-18:2ω6 levels as the regression lines converge. Thus, this abnormality may be corrected nutritionally.

Homozygotes compared to heterozygotes. The profiles of the FA composition of serum lipid classes of CF homozygotes and heterozygotes are similar, but are less pronounced in the latter. Similar deviations from normal

have also been found in the FA composition of platelet lipids of CF homozygotes and heterozygotes (41). In the PL fraction, heterozygotes and homozygotes compensate for reduced 18:2ω6 in different ways. None of our controls had 18:2ω6 levels as low as those found in CF, and how such individuals might compensate for low 18:2ω6 in their lipids could not be determined. In individuals with similarly reduced 18:2ω6 in PL due to intravenous alimentation without EFA (13), malabsorption (28) or septicemia, chronic bronchitis and other illnesses unassociated with malabsorption (42), compensation was by marked increase in monoenoic fatty acids and by a lesser increase in saturated FA. We find similar compensation in CF patients (Fig. 4). In contrast, low 18:2ω6 in parents of CF patients was compensated for entirely by increased saturated acids without any increase in monounsaturated FA. Thus, deviation from the expected pattern in EFA deficiency is found in heterozygotes but not in homozygotes.

The CF gene has recently been identified, and different mutations of this gene have been documented (42–44). These could lead to different subgroups of CF patients with different clinical and biochemical patterns (44). The putative CF gene product has been described (42), and it has been suggested that it could be an ion channel or may serve to regulate ion channel activities. The sequellae which link reduced ion channel activity with disturbed FA composition of tissue lipids, if such a link exists, is currently unknown. The heterogeneity in genetic make up of patients and carriers, due to different mutations, could contribute to the wide range in severity of FA disturbances found. The newly acquired possibility to identify different mutations enables investigation of these possibilities.

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The Lipid Composition of Selected Tissues from a Mediterranean Monk Seal, *Monachus monachus*

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The lipid composition of blubber, brain, muscle and heart from a Mediterranean monk seal *Monachus monachus* (an endangered species) were examined to allow comparisons with more common species of seals. Only neutral lipids (mainly triacylglycerols) were detectable in the blubber lipids, whereas polar lipids predominated in the heart and in the brain. Neutral and polar lipids comprised almost equal proportions in both liver and muscle. Choline glycerophospholipids (CGP) were the major polar lipids, followed by ethanolamine glycerophospholipids (EGP) in the liver, heart and muscle. Cerebrosides accounted for 28.8% of the brain lipids. All lipid classes of the liver contained high levels (31–47%) of polyunsaturated fatty acids (PUFA), with the exception of phosphatidylserine. The total proportion of n-6 PUFA exceeded that of n-3 PUFA in all lipid classes of the liver, due mainly to the high levels of 20:4n-6. The highest level of 20:4n-6 occurred in phosphatidylinositol, where it comprised 32.4% of the total fatty acids. The CGP and EGP of the brain contained lower levels of PUFA than those of the liver, muscle and heart. Alkenyl ethers accounted for 35.8% of the total long-chain moieties in brain EGP. The fatty acid composition of blubber triacylglycerols differed from those of the lipid classes from other tissues in that it had a very low ratio of n-6 to n-3 PUFA (0.3) as a result of a lower content of 20:4n-6.

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The Mediterranean monk seal, *Monachus monachus* (Hermann 1779), is one of the world's rarest mammals (1). Only a few hundred individuals remain and the species is faced with extinction (2,3). Because of its low abundance and the danger of genetic deterioration due to isolation, the monk seal is under protection in the European Community, and a seal park has been established in the Northern Aegean Sea for this purpose.

Although data exist concerning the biology, behavior and feeding habits of the Mediterranean monk seal (4), the lipid composition of its tissues has not been reported previously. In contrast, a substantial amount of information is available concerning the fatty acid composition of commercial oils, as well as tissue and milk lipids from several species of seals from Arctic and temperate waters (5–12). Most previous studies of seal lipids have concentrated on the total lipids from blubber, while the lipid composition of other tissues has not been examined in detail.

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Abbreviations: CGP, choline glycerophospholipids; CL, cardiolipin; DMA, dimethyl acetal; EGP, ethanolamine glycerophospholipids; HPTLC, high-performance thin-layer chromatography; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols.

Although data obtained to date suggest that general similarities exist in fatty acid composition between oils from different species of seal, it is known that the fatty acid composition of oils obtained from marine mammals is strongly influenced by that of the dietary lipids (7,9,11,12). The species of organisms that comprise the food webs in the Mediterranean Sea differ from those found in colder waters, and it may be expected that these differences are reflected in the lipid composition of the monk seal. For this reason, the analysis of the lipid composition of tissues from the monk seal was undertaken in the present study when the rare opportunity arose of obtaining tissues from a fresh corpse.

MATERIALS AND METHODS

Samples were collected in March 1990 from an adult (6+ years old) male *M. monachus* that was found strangled in a fisherman's net near the southern coast of Santorini Island in the Southern Aegean Sea (36°20′ N, 25°22′ E) (13). The animal was recovered about 15 h after its death and was kept surrounded by ice until its subsequent dissection at the National Centre for Marine Research (Athens, Greece). The length of the body from the tip of the snout to the tip of the tail was 218 cm, and the circumference at the navel was 121 cm. Apart from the presence of a few parasitic worms in the intestine, the seal showed no obvious indications of disease and appeared to be a healthy individual in good condition. The stomach was almost full, mainly with fish taken from the net in which it had become entangled (13). Samples of liver, brain, heart, muscle and blubber were weighed and immediately stored at −20°C in chloroform/methanol (2:1, vol/vol) under an atmosphere of nitrogen in glass vials sealed with Teflon-lined caps. Samples of blubber, taken from the belly region where the blubber was about 4 cm thick, were treated similarly. For the extraction of lipids, the samples were homogenized in chloroform/methanol (2:1, vol/vol) with subsequent washing of the organic phase with 0.88% (wt/vol) KCl, essentially as described by Christie (14). The amount of lipid recovered was determined gravimetrically after removal of the solvent by evaporation under a stream of nitrogen.

Lipid class composition was determined by subjecting aliquots of the lipid extract to high-performance thin-layer chromatography (HPTLC) alongside known standards using glass plates coated with silica gel 60 without fluorescent indicator (E. Merck, Darmstadt, Germany). Plates were first developed to half final distance with methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aqueous KCl (25:25:25:10:9, by vol) and then developed fully using hexane/diethyl

ether/glacial acetic acid (80:20:2, by vol) (15). Separated lipid fractions were detected by spraying the developed chromatogram with 3% cupric acetate in 8% phosphoric acid (16), followed by charring at 160°C for 20 min, and were quantitated by optical scanning densitometry using a Shimadzu CS-930 dual wave length scanner (Anachem, Luton, United Kingdom) linked to Shimadzu DR-2 data recorder (15).

For the analysis of fatty acid composition, aliquots of total lipid extracts were separated into component lipid classes by TLC on 20 × 20 cm glass plates coated with silica gel G60 (E. Merck) using the double development system described previously. Developed chromatograms were sprayed with 0.01% (wt/vol) 2′,7′-dichlorofluorescein in methanol containing 0.05% (wt/vol) butylated hydroxytoluene and viewing under ultraviolet light. Bands of adsorbent containing lipid classes were marked and scraped from the glass plate into test tubes. The fatty acyl moieties of the lipid classes were converted to their methyl esters by transesterification directly on the adsorbent using 1% (vol/vol) sulfuric acid in methanol for 16 h at 50°C under an atmosphere of nitrogen (14). The resultant fatty acid methyl esters were purified by HPTLC using hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) as the developing solvent and were recovered from the adsorbent by elution with hexane containing 0.05% (wt/vol) butylated hydroxytoluene as antioxidant.

Fatty acid methyl esters were analyzed by gas–liquid chromatography using a Packard 439 gas chromatograph equipped with a flame-ionization detector and fitted with a fused silica capillary column (50 m × 0.22 mm i.d.) coated with FFAP (S.G.E., Milton Keynes, United Kingdom) as the stationary phase. Hydrogen was employed as the carrier gas, and sample application was by on-column injection. The temperature of the oven was programmed to rise from 50 to 225°C during each analysis. Separated components were identified by reference to authentic standards and a well-characterized fish oil, and were quantitated by means of a Shimadzu CR6A integrator attached to the gas chromatograph.

RESULTS

The lipid contents and lipid class compositions of several tissues from the individual monk seal examined are presented in Table 1. Of the tissues analyzed other than blubber, brain had the highest lipid content (15.8%), followed in descending order by liver, muscle and heart. Only neutral lipids, mainly triacylglycerols (TAG), were detected in blubber lipid. Neutral and polar lipids comprised almost equal proportions in both liver and muscle, whereas polar lipids predominated in the brain and in the heart. TAG were the predominant neutral lipid of muscle, and in the lipids of both liver and heart, the proportions of TAG and steryl esters were similar. Cholesterol was the most abundant nuetral lipid in brain. Free fatty acids were present at a level of 10.5 to 17.0% in the total lipid extracted from all tissues except brain.

More than 70% of the lipids from the brain were polar lipids, and cerebrosides, which comprised 28% of the total lipids, were the principal lipids of this organ. Ethanolamine glycerophospholipids (EGP) were the only other class of polar lipids to comprise more than

TABLE 1

Lipid Content and Lipid Class Composition of Tissues of Mediterranean Monk Seal, *Monachus monachus*

	Liver	Brain	Heart	Muscle	Blubber
Organ weight (kg)	5.50	0.50	0.97	n.d.[a]	n.d.
Lipid content (% wet weight)	4.5	15.8	0.5	2.4	n.d
Lipid class composition (% total lipid)					
Steryl esters	10.0	0.3	3.2	6.8	—
Triacylglycerols	9.6	0.6	3.6	18.4	88.3
Free fatty acids	17.0	1.6	12.1	13.7	10.5
Cholesterol	12.3	23.5	12.8	9.1	1.2
Unidentified neutral lipids	1.3	—	1.4	0.3	—
Total neutral lipids	50.2	26.0	33.1	48.3	100.0
Cerebrosides	—	28.8	—	—	—
Sulfatides	1.8	9.4	0.6	—	—
Ethanolamine glycerophospholipids	11.7	13.3	16.4	14.0	—
Phosphatidylglycerol/cardiolipin	4.9	0.5	11.0	6.6	—
Phosphatidylinositol	7.8	2.6	6.0	5.6	—
Phosphatidylserine	3.7	7.6	4.1	1.9	—
Choline glycerophospholipids	16.7	5.8	23.8	21.4	—
Sphingomyelin	2.0	6.0	2.8	1.9	—
Lysophosphatidylcholine	1.2	—	1.4	0.3	—
Unidentified polar lipids	—	—	0.8	—	—
Total polar lipids	49.8	74.0	66.9	51.7	0.0

[a]n.d., Not determined.

10% of the brain lipids, and the proportion of sphingomyelin in the brain lipids (6%) was notably higher than in other tissues. Choline glycerophospholipids (CGP) were the major polar lipids, followed by EGP, in the liver, heart and muscle. The highest level of phosphatidylglycerol/cardiolipin (PG/CL) was observed in the lipids from heart, and phosphatidylinositol (PI) and phosphatidylserine (PS) accounted for small proportions of the lipids from all tissues except blubber. Low levels of lysophosphatidylcholine were also present in the lipids of all tissues except brain and blubber, in which they were not detectable.

High levels (31–47%) of polyunsaturated fatty acids (PUFA) were present in all liver lipids examined, with the exception of PS, in which they comprised only 19.5% of the total fatty acids (Table 2). The proportion of total n-6 PUFA always exceeded that of n-3 PUFA, due mainly to the presence of 20:4n-6 as the principal PUFA of all lipid classes except PS. A particularly high level of 20:4n-6 was observed in PI, where it accounted for over 30% of the total fatty acids. Other n-6 PUFA were only minor components of all lipid classes, with the exception of the PG/CL fraction in which 18:2n-6 comprised 30.5% of the fatty acids. This feature, combined with a low content of n-3 PUFA, conferred this fraction with a very high n-6/n-3 ratio (15.8). The principal n-3 PUFA of all lipid classes in the liver was 22:6n-3, which was most abundant in TAG (12.8%) and EGP (13.5%). Although the level of 22:5n-3 exceeded that of 20:5n-3 in all classes, both these PUFA were still only minor components. Other notable features of the liver lipid classes were the formation from EGP of around 1% each of 16:0 dimethyl acetal (DMA) and 18:0 DMA produced by transmethylation of 1-*O*-alk-1′-enyl linked ether chains and the occurrence in sphingomyelin of the highest observed level (5.4%) of 24:1n-9.

TABLE 2

Composition (wt%) of Lipid Classes from Liver of Mediterranean Monk Seal, *Monachus monachus*[a]

Long-chain moiety	TAG	CGP	EGP	PS	PI	PG/CL	SM
14:0	2.0	0.5	0.2	0.4	0.2	0.7	0.2
14:1	0.4	—	—	—	—	—	—
15:0	0.8	0.6	0.4	0.5	0.5	2.6	0.4
16:0DMA	—	0.3	1.1	—	—	—	—
16:0	16.6	15.1	6.3	11.5	3.9	3.0	10.5
16:1n-7	6.1	2.0	0.8	3.9	1.0	—	0.7
17:0	1.2	1.1	1.3	2.0	1.0	1.6	1.1
17:1	0.7	0.8	0.3	0.4	0.2	1.7	0.3
18:0DMA	—	—	1.0	—	—	—	—
18:0	6.5	26.0	31.6	41.6	33.9	3.6	27.3
18:1n-9	18.0	11.6	5.0	10.3	6.0	16.2	6.5
18:1n-7	4.7	4.7	2.2	6.6	3.1	20.3	3.1
18:2n-6	2.4	0.9	0.8	4.4	1.7	30.5	0.8
18:3n-3	1.0	—	—	—	—	—	—
20:0	—	0.2	—	—	—	—	4.7
20:1n-9	1.1	0.5	0.5	0.6	0.4	0.7	0.3
20:2n-6	0.3	0.3	0.3	0.7	1.1	4.2	0.2
20:3n-6	0.4	0.3	0.3	0.7	1.3	2.6	0.3
20:3n-3	0.2	—	0.2	—	0.3	1.6	—
20:4n-6	15.9	26.9	27.2	4.8	32.4	3.7	23.4
20:4n-3	0.5	—	0.3	—	0.4	—	—
20:5n-3	—	0.7	0.8	—	0.5	—	0.4
22:0	—	0.2	0.2	—	0.6	—	2.4
22:4n-6	1.1	0.3	1.0	1.1	1.1	—	0.3
22:5n-6	1.3	0.3	0.8	0.5	0.7	—	0.3
22:5n-3	2.6	0.7	1.9	1.4	1.2	—	0.8
22:6n-3	12.8	3.9	13.5	5.9	5.7	1.0	5.0
24:0	—	—	—	—	—	—	1.1
24:1n-9	0.3	0.4	0.1	0.6	0.2	2.3	5.4
Unidentified	3.1	1.7	1.9	2.1	2.6	3.7	4.5
Total sat.	27.1	44.0	42.1	56.0	40.1	11.5	47.7
Total mono-unsat.	31.3	20.0	8.9	22.4	10.9	41.2	16.3
Total PUFA	38.5	34.3	47.1	19.5	46.4	43.6	31.5
Total n-6	21.4	29.0	30.4	12.2	38.3	41.0	25.3
Total n-3	17.1	5.3	16.7	7.3	8.1	2.6	6.2
n-6/n-3	1.3	5.5	1.8	1.7	4.7	15.8	4.1

[a]TAG, triacylglycerols; CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; SM, sphingomyelin; sat., saturated; monounsat., monounsaturated; DMA, dimethyl acetal.

The TAG of muscle and heart were generally similar in fatty acid composition to those of liver although, unlike the latter, they both contained 20:5n-3 (Table 3). The level of PUFA in the CGP of muscle (21.2%) was lower than that of both the liver and heart due mainly to a lower content of 20:4n-6. The EGP of muscle was very similar to that of the liver in terms of PUFA content and n-6/n-3 ratio but had a higher content of alkenyl ethers as more than 18% of the long-chain moieties in muscle EGP formed 16:0 DMA, 18:0 DMA, 18:1n-7 DMA and 18:1n-9 DMA. Similar values were found for the DMAs derived from heart EGP, which was also characterized by a very high PUFA content (51%).

The fatty acid compositions of the CGP and EGP from the brain were notably different from those of the same phospholipids from other tissues (Table 4). In particular, the PUFA contents of the two brain phospholipids were much lower. Only 4.4% of the total fatty acids in brain CGP were PUFA, with the remainder comprising almost equal proportions of saturated and monounsaturated fatty acids, particularly 16:0 and 18:1n-9. The EGP of brain contained 19.9% PUFA, within which 20:4n-6 and 22:6n-3 each accounted for around 6%. The brain EGP was also characterized by its high content of alkenyl ethers. Thus, the 16:0 DMA, 18:1n-9 DMA and 18:1n-7 DMA produced 10.6, 7.1 and 12.4%, respectively, of the total long-chain moieties produced by transmethylation of this phospholipid fraction.

Of all the classes examined, the fatty acids of blubber TAG had the lowest n-6/n-3 ratio (0.3). This low ratio resulted from the fact that 20:4n-6 accounted for only 2.0% of the fatty acids in blubber TAG, whereas 22:6n-3 comprised 15.5%, the highest content of any class. The proportion of 22:5n-3 (3.4%) exceeded that of 20:5n-3 (2.0%).

DISCUSSION

The data presented in this study were obtained on only one monk seal, and, consequently, caution must be ap-

TABLE 3

Composition (wt%) of Selected Lipid Classes from Muscle and Heart of Mediterranean Monk Seal, *Monachus monachus*[a]

	Muscle			Heart		
Long-chain moiety	TAG	CGP	EGP	TAG	CGP	EGP
14:0	2.4	0.4	—	1.0	—	—
14:1	0.2	—	—	—	—	—
15:0	0.6	0.4	—	1.5	—	—
16:0DMA	—	4.6	7.2	—	8.4	4.9
16:0	11.8	27.2	4.0	18.5	19.3	3.0
16:1n-7	8.3	2.0	1.0	5.6	1.3	—
17:0	0.6	0.9	1.2	1.2	1.1	0.7
17:1	1.5	0.3	1.2	1.5	—	1.0
18:0DMA	—	0.4	4.3	—	1.1	6.0
18:1n-9DMA	—	0.7	4.5	—	2.9	5.3
18:1n-7DMA	—	0.3	2.2	—	1.0	2.8
18:0	3.5	5.7	15.9	8.7	7.5	18.8
18:1n-9	18.8	29.9	9.4	17.9	18.9	3.6
18:1n-7	3.4	4.1	1.6	3.5	4.0	1.8
18:2n-6	2.4	0.8	0.9	2.8	1.6	1.5
18:3n-3	0.8	—	—	0.5	—	—
18:4n-3	1.3	—	—	—	1.2	—
20:0	0.3	0.3	—	—	0.7	—
20:1n-9	2.6	0.4	0.6	1.9	0.8	—
20:2n-6	0.5	—	—	0.9	—	—
20:3n-6	0.2	—	—	—	—	—
20:3n-3	2.8	—	—	—	—	—
20:4n-6	14.5	14.1	25.0	6.6	23.0	39.6
20:4n-3	0.5	—	—	—	—	—
20:5n-3	2.1	1.8	2.6	2.6	2.0	3.2
22:0	1.0	0.2	—	—	—	—
22:4n-6	1.5	0.4	1.8	0.7	—	0.6
22:5n-6	1.0	0.5	1.5	1.0	—	0.5
22:5n-3	2.4	0.6	1.7	1.5	0.4	0.5
22:6n-3	10.3	3.0	12.9	14.0	1.8	5.1
24:1n-9	0.5	0.8	—	3.6	2.2	1.1
Unidentified	4.2	0.2	0.5	4.5	0.8	—
Total sat.	20.2	40.1	32.6	30.9	38.1	33.4
Total monounsat.	35.3	38.5	20.5	34.0	31.1	15.6
Total PUFA	40.3	21.2	46.4	30.6	30.0	51.0
Total n-6	20.1	15.8	29.2	12.5	24.6	42.2
Total n-3	20.2	5.4	17.2	18.1	5.4	8.8
n-6/n-3	1.0	2.9	1.7	0.7	4.6	4.8

[a]Abbreviations as in Table 2.

TABLE 4

Composition (wt%) of Selected Lipid Classes from Brain and Blubber Triacylglycerols of Mediterranean Monk Seal, *Monachus monachus*[a]

	Brain		Blubber
Long-chain moiety	CGP	EGP	TAG
14:0	1.1	—	3.7
14:1	—	—	0.6
15:0	0.8	—	0.6
16:0DMA	1.8	10.6	—
16:0	30.9	4.0	10.5
16:1n-7	4.5	1.6	13.6
17:0	0.4	0.5	0.6
17:1	0.5	0.4	2.3
18:0DMA	0.3	5.7	—
18:1n-9DMA	0.3	7.1	—
18:1n-7DMA	—	12.4	—
18:0	13.3	5.1	2.3
18:1n-9	32.0	21.4	30.2
18:1n-7	6.4	3.3	2.7
18:2n-6	0.2	—	2.0
18:3n-3	—	—	0.7
18:4n-3	0.2	—	0.3
20:0	0.2	—	0.2
20:1n-9	1.2	4.9	2.8
20:2n-6	—	—	0.7
20:4n-6	1.9	6.6	2.0
20:4n-3	—	—	0.7
20:5n-3	—	—	1.7
22:4n-6	0.3	4.8	0.9
22:5n-6	—	—	1.3
22:5n-3	0.2	2.0	3.4
22:6n-3	1.6	6.1	15.5
24:0	0.2	0.9	—
24:1n-9	1.3	1.9	0.3
Unidentified	0.4	0.7	0.4
Total sat.	49.0	26.8	17.9
Total monounsat.	46.2	53.0	52.5
Total PUFA	4.4	19.5	29.2
Total n-6	2.4	11.4	6.9
Total n-3	2.0	8.1	22.3
n-6/n-3	1.2	1.4	0.3

[a]Abbreviations as in Table 2.

plied when extrapolating the patterns of lipid composition to this species in general. The results, however, do allow a representative comparison of the lipid compositions of different tissues within an individual monk seal, a subject for which comparative data are surprisingly rare as previous studies of seal lipids have concentrated on blubber (5,6,12).

The lipid contents of blubber, heart and liver of the individual monk seal examined here are all consistent with those reported previously for other species of seal (5,6), and the high lipid content of the brain is typical of brain tissue of mammals in general (17). With the exception of the brain, all the tissues of the monk seal contained significant amounts of free fatty acids in their total lipid. Because fatty acids are not usually found to any great extent in tissues in their free, nonesterified form, this suggests that some hydrolysis of lipids had occurred *post mortem,* and, consequently, the lipid compositions of the tissues in the living animal may have been slightly different from those recorded here. Nevertheless, tissue-specific patterns of lipid class composition were still discernible, as exemplified by the high levels of cerebrosides and PG/CL observed in the lipids of the heart and brain tissues, respectively. As CL is a specific component of inner mitochondrial membranes (18), the overall lipid class composition of the heart presumably reflects the involvement of large numbers of mitochondria for the supply of energy for contraction.

The brain lipids of the monk seal examined here have many of the characteristic feature found in other mammals (17), as well as in fish (19). The fact that the value found here for the cerebroside content of seal brain

lipids (28.8%) is actually higher than that found in other mammals may be due to the sample of brain analyzed, which consisted largely of white matter, as this tissue contains a much higher concentration of cerebrosides than does gray matter (17). Further evidence in support of this statement comes from the observation that the PUFA contents of the CGP and EGP are very similar to those observed for the same phospholipids in the white matter of normal human brain (20), and, overall, the fatty acid compositions of the seal CGP and EGP more closely resemble those of human white matter than gray matter (17).

The PUFA content of the blubber TAG from the monk seal (29%) examined in the present study is similar to values that have been reported for the blubber lipids of grey seals (*Halichoerus grypus*) (5) and harbor seals (*Phoca vitulina*) (6) from Nova Scotia, and oils prepared from harp seals (*Pagophilus groenlandica*) (7) and Uruguayan fur seals (*Arctocephalus australis* Zimmermann) (10). Higher levels of PUFA have been observed in the blubber lipids of phocid seals from the Bering Sea (12) and the Baltic (11). The proportions of 20:4n-6 and 22:5n-6 observed in the blubber lipids of the monk seal examined here are, however, notably higher than those commonly found in other seal species.

When the fatty acid compositions of lipids of different tissues from the monk seal are compared, it is apparent that the blubber TAG are richer in n-3 PUFA and poorer in n-6 PUFA, particularly 20:4n-6, than the lipids of the other tissues examined. It has been shown previously that the blubber lipids of the harbor seal also contain lower levels of 20:4n-6 than the phospholipids and TAG of heart and lung (8), and that 20:4n-6 accounts for around 17% of the fatty acids in the liver of the Baltic ringed seal (*P. hispida botanica*) (11), but is almost absent in the blubber lipid. Such observations, along with the data obtained in the present study, suggest that a general pattern exists for seals in which blubber TAG are characterized by very low ratios (0.3 or less) of n-6 to n-3 PUFA, whereas in the TAG and phospholipid of internal organs the ratio is much higher, reflecting the general predominance of n-6 PUFA in these lipids. This marked difference in PUFA composition between the TAG of blubber and those of the other tissues may reflect a situation in which the bulk of fatty acids present in blubber are those that have been absorbed from the diet and transported directly in chylomicrons to the blubber for deposition without modification. At the same time, the PUFA in the lipids of other tissues may have been processed by the liver and undergone desaturation and elongation prior to their esterification. Given such a situation, the high levels of n-3 that occur in the blubber lipids of seals in these ecosystems (7) can be expected as n-3 PUFA predominate by far over those of the n-6 series in the lipids of phytoplankton, zooplankton and fish that constitute the marine food webs in temperate and polar latitudes (21). In evolutionary terms, seals are related to terrestrial mammals and as such are presumably capable of converting dietary 18:2n-6 to 20:4n-6. Such a conversion might account for the high levels of 20:4n-6 observed in the lipids of seal tissues other than the blubber. However, it is notable that the liver and muscle TAG of the monk seal examined in this study contained higher levels of 20:4n-6, but lower levels of 18:2n-6, than those of terrestrial mammals fed plant or fish oils (22,23). Assuming that the blubber fatty acid composition is a direct reflection of that of the dietary lipids, then the low level of 18:2n-6 present in the blubber lipid would suggest that large levels of this PUFA had not been available in the diet of the monk seal as a precursor of 20:4n-6. This may also imply that the monk seal had been very active in the specific conversion of 18:2n-6 to 20:4n-6. It is also possible that the monk seal had received preformed 20:4n-6 in its diet. The level of 20:4n-6 observed here for the blubber TAG suggests that the diet consumed by the individual studied contained higher levels of this PUFA than seals from more northerly latitudes. The diet of *M. monachus* in the Mediterranean Sea normally consists of fish, cephalopods and marine plants, including zostera and sargassum (4). Although the lipids of fish in general are characterized by high levels of n-3 PUFA, 20:4n-6 is more common in the lipids of both freshwater and marine fish from Australian and other tropical inshore waters (24–26). Particularly high levels of 20:4n-6 have been noted in the lipids of omnivorous fish, which consume macroalgae in these locations as part of their diet (27). It is known that 20:4n-6 is the predominant PUFA in the lipids of *Sargassum* species of algae from different parts of the world (28), and, assuming this holds true for *Sargassum* species in the Mediterranean Sea, then this macroalgae may be a direct source of this fatty acid for the monk seal. The extent to which high levels of 20:4n-6 are characteristic of organisms at all trophic levels in the Mediterranean remains to be examined.

It must be stressed that no definite conclusions should be drawn in regard to the lipid composition of a species in general from the analysis of a single animal as analyzed in this study. Nevertheless, the results obtained here indicate that the fatty acid compositions of blubber and other tissues of the Mediterranean monk seal may differ somewhat from those of seals from more temperate waters, particularly with regard to the ratio of n-6 to n-3 long-chain PUFA.

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Metabolism of Long-Chain Fatty Acids, Alcohols and Alkylglycerols in the Fish Parasite *Paratenuisentis ambiguus* (Acanthocephala)

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Specific differences between the acyl composition of lipids of the helminth *Paratenuisentis ambiguus* and its host eel, as shown previously, prompted us to study the lipid metabolism in this intestinal fish parasite. Adults and larvae of *P. ambiguus* were fed various lipid precursors, e.g., fatty acids, long-chain alcohols and 1-*O*-alkylglycerols, which may occur as common nutrients of intestinal parasites. Incorporation of [1-^{14}C]palmitic acid into neutral and polar lipids was found to be similar under aerobic and near-anaerobic conditions. In adult parasites maintained in culture medium supplemented with glucose, [1-^{14}C]palmitic acid was incorporated mainly into triacylglycerols and phosphatidylcholines, whereas [1-^{14}C]oleic acid was incorporated preferentially into triacylglycerols. In fasted adults, as well as in larvae, [1-^{14}C]oleic acid was mainly transferred to phosphatidylcholines. Lipolytic activity was detected in adult parasites that had been incubated with radioactive trioleoylglycerol. [1-^{14}C]Hexadecan-1-ol was oxidized in *P. ambiguus* at a high rate to labeled palmitic acid, which was incorporated into various lipid classes of *P. ambiguus*. Small but significant proportions of radioactivity from hexadecan-1-ol were incorporated into ether glycerolipids of the parasite. A more direct precursor in ether glycerolipid metabolism, i.e., *rac*-1-*O*-[1′-^{14}C]hexadecylglycerol, was incorporated into alkyl and 1′-alkenyl moieties of choline and etha-nolamine etherglycerophospholipids of *P. ambiguus* in high yield. High proportions of labeled diacylglycerols, triacylglycerols and steryl esters were detected in surface lipids as well as lipid extracts of the culture media after incubation of *P. ambiguus* with [1-^{14}C]palmitic or [1-^{14}C]oleic acids. The results suggest that palmitic acid and oleic acid are incorporated into neutral and polar lipids of *P. ambiguus* maintained in glucose medium quite differently with oleic acid showing a strong preference for triacylglycerols. However, the incorporation of palmitic acid in glucose-fed parasites was similar to that of oleic acid in fasted parasites, as well as in larvae. This may be explained by partial fatty acid depletion in fasted worms and rapid cell division in larvae, respectively.

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Paratenuisentis ambiguus (Acanthocephala) is a helminth that resides in the intestine of the American and the European eel (1–3). The parasites attach themselves to the wall of the intestine where they cause inflammatory reactions. This inflammation, together with the high worm burdens, is harmful to the eel leading to retarded growth of this commercially important fish. The growing interest in fish parasitology resulting from the expanding commercial aquaculture of edible fish prompted us to study the lipids of *P. ambiguus* in comparison with infected eel intestine (4,5) in order to establish differences between the lipids of parasite and host.

Helminths are able to absorb fatty acids and other lipids and to incorporate them into various lipid classes (6–8). As a consequence, the acyl compositions of the lipids of the parasites would be expected to resemble those of the host tissue. The composition of the acyl moieties of various lipids of *P. ambiguus*, however, was found to be remarkably different from that of infected eel intestine (4,5). In particular, much higher proportions of saturated long-chain fatty acids, as well as eicosapentaenoic acid, have been found in neutral and polar lipid classes of the parasite when compared to those of eel. The reason for these differences is presently not known.

In the present investigation, we have fed both adult and larval forms of *P. ambiguus* various lipid precursors which may occur as common nutrients inside the eel gut and in the coelum of the larva's host, i.e., *Gammarus tigrinus* (Amphipoda) (1), respectively, in order to shed light on lipid biosynthesis and metabolism at different developmental stages of this helminth. Studies on the regulation of lipid metabolism in *P. ambiguus* could be a useful first step toward the control of acanthocephalans in fish and of endoparasites in general.

MATERIALS AND METHODS

Chemicals, preparation of substrates and derivatization. High-performance liquid chromatography (HPLC)-grade solvents were purchased from E. Merck (Darmstadt, Germany). Fatty acid methyl ester standards were products of Nu-Chek-Prep (Elysian, MN). Methyl esters derived from acyl lipids were prepared by transmethylation using methanol/benzene/sulfuric acid (84:10:4, by vol) (9); free fatty acids (FA) were converted to their methyl esters using diazomethane (9,10). 1-*O*-Alkylglycerols were prepared following the procedure of Baumann and Mangold (11). Acetylations were carried out in acetic anhydride/pyridine (10).

Radiochemicals and preparation of radioactive substrates. [1-^{14}C]Palmitic acid (2.18 GBq/mmol), [1-^{14}C]-oleic acid (2.07 GBq/mmol), and tri[1-^{14}C]oleoylglycerol (2.07 GBq/mmol) were purchased from Amersham-Buchler (Braunschweig, Germany). Methyl [1-^{14}C]-palmitate (1.0 GBq/mmol) was derived from palmitic acid by reaction with diazomethane. [1-^{14}C]Hexadecan-1-ol

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Abbreviations: CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids; FA, free fatty acids; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; NAD+, nicotinamide adenine dinucleotide, oxidized; RP-HPLC, reverse-phase high-performance liquid chromatography; TLC, thin-layer chromatography.

(1.0 GBq/mmol) was prepared from methyl [1-^{14}C]palmitate by reduction with lithium aluminum hydride (10). *rac*-1-*O*-[1′-^{14}C]Hexadecylglycerol (0.97 GBq/mmol) was prepared from [1-^{14}C]hexadecan-1-ol and *rac*-isopropylideneglycerol (Aldrich, Steinheim, Germany) as mentioned previously (11).

Biological materials. Eels (*Anguilla anguilla*) naturally infected with *P. ambiguus* were caught in the Weser river; they were free of other intestinal helminths. *Paratenuisentis ambiguus* worms were obtained and maintained as described previously (4). In all experiments, pooled samples of the whole organisms of *P. ambiguus*, males as well as females, were used for incubations. To study lipid metabolism in fasted parasites, *P. ambiguus* worms from eels were kept for three days in modified minimum essential medium (MEM) (Flow Laboratories, Meckenheim, Germany) without glucose and serum. *Paratenuisentis ambiguus* worms survived for about one week when starved under these conditions. Larvae of *P. ambiguus* were obtained from experimentally infected *G. tigrinus* as described previously (12). At 40 d post infection, the amphipods were squashed, and the infective larvae were washed with MEM and used for assay.

Time course of [1-^{14}C]palmitic acid incorporation into the lipids of adult parasites. About 225 *P. ambiguus* worms (average fresh weight 2.5 mg/animal) were incubated at room temperature with 2 μCi (20 mCi/mmol) [1-^{14}C]palmitic acid (ammonium salt) in 10 mL modified MEM, either under aerobic or nearly anaerobic (nitrogen atmosphere) conditions. Aliquots of worms were removed from the medium after 1, 6 and 24 h and washed with MEM. After homogenization of the worms (around 200 mg fresh weight, each) in dichloromethane/methanol (1:2, vol/vol), using an Ultra-Turrax blender (IKA-Werke, Staufen, Germany), total lipids were extracted as described below.

Incubation of P. ambiguus, *adults and larvae, as well as fasted worms, with various ^{14}C-labeled substrates.* Adult and larval parasites, about 100 animals each, were incubated in 5 mL modified MEM with 2 μCi (20 mCi/mmol) radioactively labeled substrates, i.e., [1-^{14}C]palmitic and [1-^{14}C]oleic acids (ammonium salts), tri[1-^{14}C]oleoylglycerol, [1-^{14}C]hexadecan-1-ol or *rac*-1-*O*-[1′-^{14}C]hexadecylglycerol, for 24 h under aerobic conditions. Substrates were added in ethanolic solution (50 μL). Fasted worms were incubated in modified MEM without glucose and serum. Total lipids of adult worms and larvae were extracted as described in the next paragraph(s).

Isolation of surface lipids. After incubation with various substrates, e.g., radioactive fatty acids, *P. ambiguus* worms were taken from the medium by forceps and washed with MEM. Adhering medium was removed with tissue paper, and the surface lipids were extracted by gentle shaking for about 20 s in dichloromethane/methanol (98:2, vol/vol) according to a method adapted from Holloway (13).

Extraction of total lipids. Paratenuisentis ambiguus worm tissue (200–300 mg fresh weight, freed of surface lipids as described above, was homogenized in 4 mL dichloro-methane/methanol (1:2, vol/vol). The total lipid extract was obtained as described previously (4). Total lipids from culture media were extracted in a similar manner with 4 mL of dichloromethane/methanol (1:2, vol/vol) per mL medium.

Lipid analysis. Aliquots of total lipids were separated into various lipid fractions, e.g., wax esters, steryl esters, triacylglycerols, long-chain aldehydes, FA, choline (CPL) and ethanolamine glycerolipids (EPL), as well as sphingomyelins, by preparative thin-layer chromatography (TLC) on Silica Gel H (E. Merck), and the various lipid fractions were purified and derivatized as described previously (4,5). The fraction of long-chain aldehydes isolated from *P. ambiguus* after incubation with [1-^{14}C]hexadecan-1-ol (see Table 3 later) was purified by re-chromatography on Silica Gel H layers (0.3 mm) with hexane/diethyl ether (85:15, vol/vol) as solvent and identified by co-chromatography with a standard. The aldehyde fraction was isolated and reduced with $LiAlH_4$ (as already described). The resulting long-chain alcohols were acetylated (10) and the alkyl acetates identified by radio-reverse-phase (RP) HPLC, as described later. All lipid fractions on TLC plates were identified by co-chromatography with standards and detected by brief exposure to iodine vapors. In addition, the fractions of phospholipids were detected by staining with specific reagents as described previously (5).

Triacylglycerol fractions derived from incubations of *P. ambiguus* with tri[1-^{14}C]oleoylglycerol, as well as the fatty acid methyl esters derived from total lipids of parasites after incubation with [1-^{14}C]palmitic or [1-^{14}C]oleic acids, were fractionated according to their number of double bonds by argentation-TLC (10) on layers of Silica Gel G (E. Merck) containing 25% $AgNO_3$. The plates were developed three times with toluene at 5°C for the separation of fatty acid methyl esters and with dichloromethane/methanol (99:1, vol/vol) for the separation of triacylglycerols.

Aliquots of triacylglycerols were subjected to hydrolysis with lipase from porcine pancreas (Boehringer-Mannheim, Mannheim, Germany), and the resulting reaction products were separated by TLC (10). The radioactivity in the various fractions, i.e., mono- and diacylglycerols as well as FA was determined by scanning. The fractions of 2-acylglycerols (representing the *sn*-2 fatty acids) and the FA (from *sn*-1 plus *sn*-3 positions) were used for the determination of the positional distribution of labeled fatty acids. Labeled fatty acid methyl esters were prepared from the various hydrolysis products as described above and analyzed by radio-RP-HPLC.

Aliquots of CPL and EPL were hydrolyzed by phospholipase A_2 from porcine pancreas (Boehringer-Mannheim), and the resulting reaction products were separated by TLC (10). The products of hydrolysis, i.e., FA of *sn*-2 position as well as 1-acyl-2-lysophospholipids (representing the *sn*-1 fatty acids), were isolated, derivatized to the corresponding methyl esters and analyzed by radio-RP-HPLC as described below. Aliquots of total lipids were reduced with $LiAlH_4$. The resulting mixtures of long-chain alcohols and 1-*O*-alkylglycerols plus 1-*O*-(1′-alkenyl)glycerols were separated as described previously (4) and finally analyzed by radio-RP-HPLC as described below.

Determination of radioactivity. The distribution of radioactive fractions on thin-layer chromatograms was de-

termined with a Berthold LB 2832 Automatic TLC-Linear Analyzer in combination with an LB 500 data system (EG&G Berthold, Wildbad, Germany). Solutions were mixed with Toluene Scintillator (Packard, Frankfurt/M., Germany) or Aquasol-2 (NEN, Dreieich, Germany), and radioactivity was determined by liquid scintillation counting using an LKB-Wallac 1214 Rackbeta instrument (LKB, Stockholm, Sweden).

A Merck-Hitachi L-6200 pump equipped with an ACS mass detector model 750/14 (Applied Chromatography Systems, Macclesfield, United Kingdom) and with a Berthold LB 506 B HPLC Radioactivity Monitor in combination with a Splitter Mixer LB 5034 (EG&G Berthold) was used for radio-HPLC; and a Merck-Hitachi D-2500 Chromato Integrator for monitoring mass and radioactivity traces. Labeled lipids and lipid derivatives were analyzed by RP-HPLC on two LiChrospher 100 RP-18 (5 µm) columns (E. Merck) in sequence (125 × 4 mm, each) using acetonitrile or acetonitrile/acetone mixtures as eluents. Toluene scintillator (Packard) was continuously added to the solvent stream in a ratio of 3:1 (vol/vol). Triacylglycerols were resolved with acetonitrile/acetone (62:38, vol/vol) at a flow rate of 1.0 mL/min (14). Fatty acid methyl esters and alkyl acetates were separated with acetonitrile at a flow rate of 0.6 mL/min. 1-*O*-Alkyldiacetylglycerols dissolved in acetone were injected for ether glycerolipid analysis. The mixtures of 1-*O*-alkyldiacetylglycerols derived from total lipids of *P. ambiguus* tissue were separated with acetonitrile at a flow rate of 0.8 mL/min. Synthetic standards were used for comparison.

RESULTS AND DISCUSSION

The various lipid precursors with which the adult *P. ambiguus* endoparasites were incubated *in vitro* can be expected to occur in eel intestine as possible nutrients for the parasites. For example, fatty acids and alkylglycerols are formed by intestinal lipolysis of acyl glycerolipids and alkyl glycerolipids (ether lipids), respectively, while long-chain alcohols are known to be common constituents of aquatic animals (15) that fall prey to eel. In a similar manner, fasted adults, as well as larvae of *P. ambiguus*, were incubated with fatty acids in order to study lipid metabolism at different physiological and developmental stages of this parasite. To our knowledge, the biosynthesis of neutral and polar lipids in acanthocephalans by use of labeled fatty acids and other lipid precursors, except for [1-^{14}C]acetate (16,17), has not been reported previously.

Metabolism of [1-^{14}C]palmitic and [1-^{14}C]oleic acid in P. ambiguus. The time course of incorporation of radioactivity from [1-^{14}C]palmitic acid into the lipids of adult *P. ambiguus* parasites was studied under aerobic and nearly anaerobic conditions, the latter being similar to the intestinal environment. Table 1 shows that in both incubations the incorporation of radioactivity into the total lipids, as well as the distribution of radioactivity in the various lipid classes, were similar. After a period of 24 h, most of the radioactivity was concentrated in phosphatidylcholines (around 40%) and triacylglycerols (around 20%), whereas minor proportions of radioactivity were detected in diacylglycerols and phosphatidylethanol-amines as well as steryl esters and wax esters. These data show that the metabolism of palmitic acid in adult *P. ambiguus* parasites is not specifically affected by oxygen. Similar results have been obtained for the incorporation of radioactive acetate into the neutral and polar lipids of the cestodes *Hymenolepsis diminuta* and *Spirometra mansonoides* under aerobic and anaerobic conditions (16,17).

When the incorporation of radioactivity from [1-^{14}C]-palmitic acid was compared to that from [1-^{14}C]oleic acid, different patterns were observed for the incorporation of palmitoyl and oleoyl moieties into the various lipid classes of adult *P. ambiguus* (Table 2). Again, palmitic acid was esterified predominantly into triacylglycerols and phosphatidylcholines, whereas oleic acid was incorporated mainly into triacylglycerols (81%). Small proportions of radioactivity of both labeled fatty acids were found to be channelled into diacylglycerophosphoethanolamines as well as steryl esters. Moreover, it is obvious from the incorporation of radioactivity into total lipids as well as from the relatively high concentration of unmetabolized [1-^{14}C]palmitic acid and the low concentration of [1-^{14}C]oleic acid that oleic acid was esterified into various lipid classes more extensively than was palmitic acid (Tables 1 and 2).

TABLE 1

Time Course of the Incorporation of Radioactivity into Various Lipid Classes of Adult *Paratenuisentis ambiguus* Worms After Incubation with [1-^{14}C]Palmitic Acid Under Aerobic and Anaerobic Conditions[a]

Incubation conditions	Time (h)	Radioactivity (pmoles/animal) in TL[a,b]	Distribution of radioactivity (%) in various lipid fractions								
			SM	PC	PE	Other polar lipids	DAG	FA[c]	TAG	SE	WE
Aerobic	1	27	trace[d]	11	4	2	11	65	3	trace	trace
	6	100	4	37	2	3	3	25	18	4	4
	24	133	trace	41	3	5	2	17	18	7	6
Anaerobic	1	15	trace	20	4	4	4	63	trace	trace	trace
	6	60	trace	26	3	3	4	42	13	5	4
	24	180	trace	42	3	4	trace	20	16	7	6

[a]TL, total lipids; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; TAG, triacylglycerol; SE, steryl esters; WE, wax esters; FA, free fatty acids. [b]Excluding radioactivity of FA. [c]Substrate. [d]Trace (<2%).

TABLE 2

Incorporation of Radioactivity into Various Lipid Classes of Adult *Paratenuisentis ambiguus* Worms After Incubation with [1-^{14}C]Palmitic Acid and [1-^{14}C]Oleic Acid as well as of Adult Fasted Worms and Larvae with [1-^{14}C]Oleic Acid[a]

Substrate/physiological or developmental stage	Radioactivity (pmoles/animal) in TL[b]	Distribution of radioactivity (%) in various lipid fractions								
		SM	PC	PE	Other polar lipids	DAG	FA[d]	TAG	SE	WE
Palmitic acid/adults	93	trace[d]	30	2	3	7	33	20	2	2
Oleic acid/adults	303	trace	7	trace	trace	6	3	81	trace	trace
Oleic acid/fasted adults	220	4	30	6	3	trace	9	30	10	6
Oleic acid/larvae	101	trace	66	9	5	6	trace	9	2	trace

[a]Abbreviations as in Table 1. [b]Excluding radioactivity of FA. [c]Substrate. [d]Trace (<2%).

The metabolism of [1-^{14}C]oleic acid in parasites of different physiological status, i.e., maintained in glucose-supplemented or glucose- and serum-free modified MEM ("fasted"), was found to be completely different (Table 2). In *P. ambiguus* worms that were kept in glucose-supplemented medium, [1-^{14}C]oleic acid was incorporated almost exclusively into triacylglycerols. In parasites that were maintained in glucose- and serum-free modified MEM, however, labeled oleoyl moieties were found predominantly in both triacylglycerols (33%) and phosphatidylcholines (33%), as well as in a number of other neutral and polar lipids, e.g., steryl esters, wax esters, sphingomyelins and phosphatidylethanolamines.

In larvae of *P. ambiguus*, [1-^{14}C]oleic acid was esterified mainly into polar lipids, particularly phosphatidylcholines (66%), whereas only small proportions of radioactivity were found in triacylglycerols (9%) (Table 2). Thus, the incorporation of labeled oleic acid into the lipids of larvae is similar to that in fasted adult parasites.

Wax esters are widely distributed in the animal and plant kingdom and occur in high proportions in many aquatic and predominantly in marine animals. Small proportions of wax esters have been detected in *P. ambiguus* as well (4), whereas infective larvae of the acanthocephalan *Polymorphus minutus* were found to contain high proportions of these apolar lipids (18).

Both adults and larvae of *P. ambiguus* incorporated only small proportions (<2–6% of incorporated radioactivity) of labeled palmitic and oleic acid into wax esters (Tables 1 and 2). Even when adult *P. ambiguus* worms were incubated with [1-^{14}C]hexadecan-1-ol, which is a direct precursor of wax ester biosynthesis, only 8% of the label incorporated was recovered in wax esters (Table 3). From these data it may be concluded that wax ester biosynthesis is of minor importance in both adults and larvae of *P. ambiguus*. In this context, it is of interest that wax esters do not play a striking role as surface lipids of the parasite either.

The observation that palmitic acid and oleic acid were esterified into the lipids of *P. ambiguus* in a different manner is interesting. Fasted worms and larvae have low amounts of surplus fatty acids at their disposal because of the minor nutrient supply and rapid cell division, respectively, as compared to adult parasites maintained under standard conditions. This may explain the preferred incorporation of exogenous fatty acids into membrane lipids, e.g., phosphatidylcholines and phosphatidylethanolamines, as compared to storage lipids (triacylglycerols), at these two physiologically or developmentally different stages of *P. ambiguus* (Table 1).

Stereospecific distribution of acyl moieties in various glycerolipids. Table 4 shows the stereospecific distribution of radioactively labeled acyl moieties between the different positions of the glycerol backbone of triacylglycerols and phosphatidylcholines of *P. ambiguus*, adults and larvae, after incubation with [1-^{14}C]palmitic and [1-^{14}C]oleic acid, respectively. The data indicated that labeled palmitic acid was incorporated mainly into the *sn*-1 or *sn*-3 positions of triacylglycerols and into the *sn*-1 position of phosphatidylcholines of adult parasites. Similar results have been obtained for saturated long-chain fatty acids in endogenous phosphatidylcholines from *P. ambiguus* (N. Weber, K. Aitzetmüller, and H. Taraschewski, unpublished data).

TABLE 3

Incorporation of Radioactivity into Various Lipid Classes of Adult *Paratenuisentis ambiguus* Worms After Incubation with [1-^{14}C]Hexadecan-1-ol and *rac*-1-*O*-[1-^{14}C]Hexadecylglycerol[a]

Substrate/developmental stage	Radioactivity (pmoles/animal) in TL[b]	Distribution of radioactivity (%) in various lipid fractions											
		SM	PC	PE	Other polar lipids	DAG	AG[c]	ROH[c]	FA	ALD	TAG	SE	WE
Hexadecan-1-ol/adults	302	trace[d]	32	10	trace	9	—	trace	trace	4	16	16	8
1-*O*-Hexadecylglycerol/adults	150	trace	27	6	trace	trace	57	—	trace	—	trace	trace	trace

[a]Abbreviations as in Table 1. AG, 1-*O*-alkylglycerols; ROH, long-chain alcohols; ALD, long-chain aldehydes. [b]Excluding radioactivity of substrates. [c]Substrate. [d]Trace (<2%).

TABLE 4

Stereospecific Distribution of Radioactive Acyl Moieties Between the Different Positions (*sn*-1, *sn*-2 and *sn*-3) at the Glycerol Backbone of Triacylglycerols and Phosphatidylcholines of *Paratenuisentis ambiguus*, Adults and Larvae, After Incubation with [1-^{14}C]Palmitic Acid or [1-^{14}C]Oleic Acid[a]

Substrate/physiological or developmental stage	Lipids[a]	Position at glycerol	Radioactivity (%) in acyl moieties
Palmitic acid/adults	TAG	*sn*-1,3	78
		sn-2	22
	PC	*sn*-1	67
		sn-2	33
Oleic acid/adults	TAG	*sn*-1,3	45
		sn-2	55
	PC	*sn*-1	40
		sn-2	60
Oleic acid/fasted adults	TAG	*sn*-1,3	95
		sn-2	5
	PC	*sn*-1	59
		sn-2	41
Oleic acid/larvae	TAG	*sn*-1,3	90
		sn-2	10
	PC	*sn*-1	53
		sn-2	47

[a]Abbreviations as in Table 1.

Labeled oleoyl moieties, however, were almost equally distributed between the *sn*-1,3 and *sn*-2 positions of triacylglycerols, as well as the *sn*-1 and *sn*-2 positions of phosphatidylcholines in adult *P. ambiguus* worms. Our results are in agreement with the stereospecific distribution of palmitoyl and oleoyl moieties found in the lipids, particularly phospholipids, of vertebrates (19,20).

In contrast to the above pattern of stereospecific distribution in adult parasites, [1-^{14}C]oleoyl moieties were preferentially transferred to the *sn*-1 and/or *sn*-3 positions of triacylglycerols in fasted adults (maintained in glucose- and serum-free modified MEM), as well as in larvae of *P. ambiguus*. The incorporation of labeled oleic acid into the phosphatidylcholines of larvae, however, was similar to that found in adult *P. ambiguus* worms which had been incubated under standard conditions (glucose-supplemented MEM).

These results also show that physiologically or developmentally different stages of *P. ambiguus*, such as fasted adults as well as larvae, are able to esterify oleic acid into specific positions of the glycerol backbone. Similar results are known from experiments with fish, indicating that both the quantity of fatty acids transferred to, as well as their stereospecific distribution at the glycerol molecule, depend on the diet offered to the animals (20).

Metabolism of [1-^{14}C]hexadecan-1-ol and rac-*1-O-[1-^{14}C]hexadecylglycerol: Formation of ether glycerolipids.* In animal tissues, acyl-CoA thioesters are reduced to the corresponding long-chain alcohols *via* long-chain aldehyde intermediates (21). Saturated and monounsaturated long-chain alcohols are precursors of ether glycerolipids (22,23). They can also be acylated to yield wax esters or can be reoxidized to fatty acids which, in turn, can be incorporated into various ester lipids (24). Adult *P. ambiguus* worms were incubated with [1-^{14}C]hexadecan-1-ol in order to study the metabolism of this long-chain alcohol and in particular, its use in the formation of ether glycerolipids. After a 24-h incubation under aerobic conditions, most of the radioactive substrate was oxidized to the corresponding fatty acid, i.e., palmitic acid, which was incorporated into various ester lipids in a similar manner as found in the incubations with [1-^{14}C]palmitic acid. Only small proportions of radioactivity from hexadecan-1-ol were found to be incorporated into ether glycerolipids

TABLE 5

Distribution of Radioactivity in 1-*O*-Alkyl-, 1-*O*-Alkenyl- and Acyl Moieties of Various Lipid Fractions from *Paratenuisentis ambiguus*, Adults and Larvae, After Incubation with Various Radioactive Lipid Precursors

		Distribution of radioactivity (%) in various radyl moieties		
Substrate/developmental stage	Lipids[a]	1-*O*-Alkyl	1-*O*-(1′-Alkenyl)	Acyl
Palmitic acid/adults	TRG	22	trace[b]	78
	CPL	2	trace	98
Oleic acid/adults	TRG	5	trace	95
	CPL	trace	trace	99
Oleic acid/larvae	TRG	7	trace	93
	CPL	trace	trace	99
Hexadecan-1-ol/adults	TRG	23	trace	77
	CPL	7	trace	93
	EPL	14	trace	86
1-*O*-Hexadecylglycerol/adults	CPL	77	11	12
	EPL	90	8	trace

[a]TRG, triradylglycerols; CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids.
[b]Trace (<2%).

(Table 5). For example, the proportion of radioactive 1-*O*-alkyl moieties in the fraction of triradylglycerols was 23%, whereas CPL and EPL contained 7 and 14% of 1-*O*-[1-^{14}C]alkyl moieties, respectively. The radioactivity in the alkyl moieties was almost equally distributed between neutral and polar ether lipids. Relatively high proportions of labeled steryl esters and wax esters were detected in the lipids of parasites incubated with [1-^{14}C]hexadecan-1-ol. The occurrence of small proportions of radioactivity (4%) in the fraction of long-chain aldehydes (Table 3), which are known to be intermediates of the oxidation of long-chain alcohols in vertebrates, confirms the involvement of this intermediate (21,25). The extent of ether lipid formation from [1-^{14}C]hexadecan-1-ol was in a similar range as it was found for the incubations with [1-^{14}C]palmitic acid (Table 5). Incubations of adult and larval *P. ambiguus* parasites with [1-^{14}C]oleic acid, however, led to ether glycerolipids only in small proportions (Table 5).

In vertebrates, the oxidation of long-chain alcohols to the corresponding fatty acids *via* long-chain aldehydes depends on the concentration of nicotinamide adenide dinucleotide (NAD^+) in the cells (21). The reactions are catalyzed by a long-chain alcohol: NAD^+ oxidoreductase, and the sequence of reactions is reversible in the presence of surplus NADH (NAD^+, reduced) (25). An enzyme having similar activities may be responsible for the metabolism of long-chain alcohols in *P. ambiguus*. It is of interest that various oxidoreductase activities were reported of two acanthocephalans, i.e., *P. minutus* and *Moniliformis moniliformis* (26,27). However, the specificity of the latter enzymes for long-chain alcohols had not been previously investigated. Oxygen which is necessary for the maintenance of the reaction may be present in eel intestine in sufficient concentration or may be available for the parasite from the host's blood.

1-*O*-Alkylglycerols are more direct precursors of complex ether lipid biosynthesis than are long-chain alcohols; *rac*-1-*O*-[1-^{14}C]hexadecylglycerol was therefore fed to adult *P. ambiguus* worms. In contrast to long-chain alcohol, the latter substrate was converted predominantly to 1-*O*-hexadecyl-2-acylglycerophosphocholines (Table 5). In addition, small proportions of radioactivity were found in 1-*O*-hexadecyl-2-acylglycerophosphoethanolamines and 1-*O*-hexadecyl-2,3-diacylglycerols. Only trace amounts of the substrate were oxidized to fatty acids during the 24-h incubation period. It is of interest that high proportions of labeled plasmalogens were detected in the two ether phospholipid classes after incubation with radioactive 1-*O*-hexadecylglycerol.

Surface lipids and lipid extracts from culture medium. The distribution of radioactivity in the surface lipids isolated from *P. ambiguus*, in adults and larvae, after incubation with [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid and [1-^{14}C]hexadecan-1-ol, is shown in Table 6. After deduction of radioactive substrates, surface lipids usually contributed 1–2% of radioactivity of total lipids. Highest incorporation of radioactivity was detected in the neutral lipid fractions, e.g., di- and triacylglycerols, as well as in steryl esters, which were the predominantly labeled lipids of the surface coat of *P. ambiguus*. It is well known that weakly polar lipids typically occur in the surface lipids of many organisms, including helminths (28). For example, a variety of such apolar lipids form the lipid coat of parasitic nematodes (29). Labeled wax esters, however, which are known to be typical surface lipids of plants and animals were found in *P. ambiguus*, even after incubation with [1-^{14}C]hexadecanol, in trace amounts only. It is suggested that these lipids do not play an important role as surface lipids in *P. ambiguus*.

In this context it was of interest to investigate the distribution of radioactivity in lipids that had been extracted from culture media after incubation of *P. ambiguus*, adults and larvae, with various radioactive substrates. Only small proportions of lipid metabolites, predominantly neutral lipids, such as diacylglycerols, triacylglycerols and steryl esters, were detected in the culture media (Table 6). Little is known about the excretion of lipids by acanthocephalans; most probably it occurs predominantly in the region of the presoma where the incorporation of radioactive triolein has also been observed (3,8). It is not presently clear whether or not the radioactive lipids extracted from the culture media can be regarded as excretion products of *P. ambiguus* or whether they may be derived from the surface coat of the parasites.

Lipolytic activities in P. ambiguus. *Paratenuisentis ambiguus* parasites are able to hydrolyze triacylglycerols, as is evident from incubation with tri[1-^{14}C]oleoylglycerol. After an incubation period of 24 h, about 42% of the substrate had been hydrolyzed, i.e., 55 pmoles/animal. The

TABLE 6

Distribution of Radioactivity in Various Lipid Classes of Surface Lipids (SL) of *Paratenuisentis ambiguus*, Adults and Larvae, After Incubation with Various Radioactive Substrates as well as Lipids That Had Been Extracted from the Corresponding Culture Media (CM)[a]

Substrate/developmental stage	Distribution of radioactivity (%) in various lipid fractions															
	PC		PE		DAG		FA		TAG		SE		WE		ROH	
	SL	CM	SL	CM	SL	CM	SL	CM	SL	CM	SL	CM	SL	CM	SL	CM
Palmitic acid/adults	2	nd[b]	trace[c]	trace	trace	4	83[d]	94[d]	2	trace	9	trace	trace	trace	—	—
Oleic acid/adults	3	3	trace	trace	10	8	47[d]	87[d]	15	trace	20	trace	trace	trace	—	—
Oleic acid/larvae	3	4	trace	trace	4	nd	65[d]	89[d]	11	trace	12	2	trace	2	—	—
Hexadecan-1-ol/adults	6	6	trace	trace	5	7	6	trace	5	7	20	9	trace	trace	55[d]	66[d]

[a]Abbreviations as in Tables 1 and 3. [b]Not detected. [c]Trace (<2%). [d]Substrate.

resulting labeled oleoyl moieties were incorporated predominantly into triacylglycerols, as was shown for the incubations with [1-^{14}C]oleic acid. After the incubation of *P. ambiguus* worms with [^{14}C]triolein, labeled triacylglycerols were fractionated by argentation-TLC, followed by radio-RP-HPLC analysis. The results of these chromatographic analyses revealed that radioactive dioleoylpalmitoylglycerol (15%) was the main triacylglycerol species formed, together with small proportions (4%) of triacylglycerol species containing polyunsaturated acyl moieties. Triacylglycerol hydrolase activity had been found in other endoparasites, such as *Schistosoma japonicum* and *S. mansoni* (Trematoda), as well (30,31).

In conclusion, we found that the fish parasite *P. ambiguus* can absorb fatty acids from the surrounding medium and esterify them into various lipid classes, predominantly triacylglycerols and phosphatidylcholines. The pattern of fatty acid incorporation into the various lipid classes depends on the individual fatty acid substrate (saturated/monounsaturated) as well as on the developmental stage (larva/adult) and nutritional condition (glucose fed/starved) of *P. ambiguus*. Other lipid substrates, such as hexadecanol and 1-*O*-hexadecylglycerol, are also absorbed by the parasite and incorporated into ether lipids. Under aerobic conditions, 1-hexadecanol was extensively oxidized to palmitic acid. Triacylglycerol lipase activity, which may be involved in the formation of individual molecular species of glycerolipids as well as in catabolic reactions, was found in the parasite. From these results it is obvious that *P. ambiguus* is able to synthesize complex acyl and alkyl glycerolipids from fatty acids, long-chain alcohols and alkylglycerols that occur in fish intestine. Specifically, the parasite seems to channel saturated long-chain fatty acids into the phospholipids. This is consistent with the results on the acyl composition of phosphatidylcholines, phosphatidylethanolamines and phosphatidylserines of *P. ambiguus* (5). Chain elongation, particularly of palmitic acid, has also been observed in *P. ambiguus* (N. Weber, K. Aitzetmüller, and H. Taraschewski, unpublished data).

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METHOD

An Improved Spectrophotometric Triiodide Assay for Lipid Hydroperoxides

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A spectrophotometric method is described for the measurement of lipid hydroperoxides, to a lower limit of 0.5 nmoles, based on the formation of triiodide ions measured at the absorbance maximum of 365 nm. The assay mixture, which was modified from an earlier published procedure [El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A.Y., and Jürgens, G. (1989) *J. Lipid Res. 30*, 627–630], contains 18% methanol together with nonionic and cationic detergents, and is designed so that the hydroperoxides to be measured can be added in either water or methanol. By incubating the reaction mixture at 50°C for 30 min, less-reactive hydroperoxides can be measured with the same fidelity as more-reactive ones. Under these conditions, the assay can be carried out under ordinary room lighting and without special protection from ambient oxygen with absorbance values being stable up to 15 h. Enzymatic standardizations showed that the triiodide method gave comparable results for H_2O_2, cumene hydroperoxide, linoleic acid hydroperoxide, phosphatidylcholine hydroperoxide, and a photooxidized tissue extract containing a mixture of hydroperoxides. The triiodide assay is recommended primarily for measuring purified hydroperoxides.
***Lipids 29*, 591–594 (1994).**

Standard methods for the iodometric assay of hydroperoxides normally require careful exclusion of air and the use of special glassware, making it difficult and time-consuming to process multiple samples (1,2). The speed and convenience offered by the iodometric method described by El-Saadani *et al.* (3) for assaying aqueous samples of peroxidized lipoproteins prompted us to try to use it with a wide range of organic hydroperoxides. We found that within the recommended incubation time of 30 min at ambient temperature, samples of H_2O_2 gave values of absorbance at 365 nm that were consistent with the quoted molar absorptivity of I_3^- (3), but that samples of cumene hydroperoxide (CuOOH) or *t*-butyl hydroperoxide required up to 24 h of incubation time. However, by incubating samples at 50°C, as used by Gebicki and Guille (2) in their oxygen-sensitive iodometric method, the reaction was complete with a wide variety of hydroperoxides within thirty minutes.

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Abbreviations: BHT, butylated hydroxytoluene; CuOOH, cumene hydroperoxide; EDTA, ethylenediaminetetraacetic acid; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HPLC, high-performance liquid chromatography; Lin, linoleic acid; LinOOH, linoleic acid hydroperoxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PHGPX, phospholipid hydroperoxide glutathione peroxidase; TLC, thin-layer chromatography.

Additional modifications we made include the use of a more concentrated color reagent (3), in order to allow a larger sample volume, the elimination of butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid (EDTA) from the incubation mixture, and the incorporation of a standard volume of methanol which serves as a convenient solvent for measuring more lipophilic hydroperoxides.

MATERIALS AND METHODS

Materials. Peroxide-free Triton X-100 was from Calbiochem (La Jolla, CA), CuOOH was from Fluka AG (Buchs, Switzerland), dichloromethane of high-performance liquid chromatography (HPLC) grade was from Burdick and Jackson (Muskegon, MI), triphenylmethyl hydroperoxide was from Aldrich (Milwaukee, WI), hydroxylapatite was from Biorad (Hercules, CA), and silica gel HL thin-layer chromatography (TLC) plates were from Analtech (Newark, DE). Linoleic acid (Lin), soybean phosphatidylcholine (PC), rose bengal, reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), yeast glutathione reductase (GR) and bovine glutathione peroxidase (GPX) were from Sigma (St. Louis, MO). Other chemicals were from standard commercial sources. Methanol and chloroform were redistilled in glass before use.

Preparation of lipid hydroperoxides. Lipids were photooxidized in a stoppered glass cell using a modification of the procedure of van Kuijk *et al.* (4). The stirred reaction mixture contained 1.85 mL methanol, 0.1 mL rose bengal stock solution (1.25 mg Na rose bengal/mL methanol) and 0.05 mL PC stock solution (100 mg PC/mL chloroform). Illumination was for 16 h at room temperature with white light from a Tungsten-Halogen lamp focused with a glass lens onto the edge of the reaction cell. The incident energy was measured to be 2,000 W/m^2 at the surface of the cell. After illumination, the hydroperoxides were separated from the rose bengal sensitizer by adding 4 mL of dichloromethane and 2 mL of water, and the resulting lower phase was retained. The lower phase was then reextracted 2 times with 4 mL of water/methanol (1:1, vol/vol) and dried at 45°C under a stream of nitrogen. The PC hydroperoxide (PCOOH) preparations were stable for at least 6 mon when dissolved in methanol and stored at –40°C.

The PCOOH reaction product appeared as a single fraction at an R_f of 0.14 on silica gel TLC plates when developed with chloroform/methanol/water (65:35:3.5, by vol) (5) and stained for lipids (5) or peroxides (6). The hydroperoxide of linoleic acid (LinOOH), prepared and analyzed in the same way, migrated at an R_f of 0.93. The ab-

sence of a rapidly-migrating impurity in the PCOOH preparations indicated that the procedure did not result in significant hydrolysis of the fatty acid peroxides from the phospholipid. For PCOOH, the molar ratio of hydroperoxide to organic phosphate (5) was between 0.5 and 1.0, depending on the duration of illumination and on light intensity.

Enzymatic procedures. Enzymatic assay of hydroperoxides followed the procedure of Heath and Tappel (7) which is based on NADPH oxidation, monitored at 340 nm, coupled with GR to the reduction of peroxides by GSH catalyzed by either GPX (7) or phospholipid hydroperoxide glutathione peroxidase (PHGPX; Ref. 8). The reaction mixture for GPX was kept at pH 7.0, as described (9), except that the incubation was done at room temperature. Incubations using PHGPX were carried out at pH 7.4 at 35°C as described (8). Concentrations of hydroperoxides differing by a factor of two were used for all enzymatic determinations, and all assays gave proportional values. Absorbance at 340 nm was monitored until the rate of change of the experimental samples equalled that of a control containing no added hydroperoxide. The value of $6.0 \times 10^3\ M^{-1} \times cm^{-1}$ (10) was assumed for the molar absorptivity of NADPH. Partially-purified PHGPX was isolated from pig liver by acetone precipitation of a 100,000 $\times g$ aqueous supernatant, followed by column chromatography on Sephadex G-10, hydroxyl apatite and Sephadex G-75. The preparation was free of GPX, and of GSH *S*-transferase as assayed with H_2O_2 (9) and chlorodinitrobenzene (11) as substrates, respectively.

Iodometric assay. In our standard procedure, up to 30 nmoles of hydroperoxide, in either water or in methanol, were added to 10 × 75-mm glass tubes. Samples dissolved in water were brought to 0.4 mL by addition of water, followed by addition of 0.2 mL of methanol; samples dissolved in methanol were diluted to a volume of 0.2 mL, followed by 0.4 mL of water. Color reagent (0.5 mL) was added, and the contents were mixed by vortexing within 1 min after the addition. The tubes were capped with parafilm and incubated in a water bath at 50°C for 30 min. After cooling to room temperature, the absorbance at 365 nm was determined by subtracting the absorbance of a blank containing only color reagent and solvent. A stock color reagent was made up by combining 9.3 mL 1M K_2HPO_4, 30.7 mL 1M KH_2PO_4, 1.5 mL 0.02M NaN_3, 4 mL 10% peroxide-free Triton X-100, 2 mL 1% benzalkonium chloride and 4 mL of 0.5 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The stock mixture was diluted to 100 mL with water and stored at 4°C where it was stable for 6 mon. Forty mg of KI per mL of reagent was added just before use.

RESULTS AND DISCUSSION

Excellent proportionality between added hydroperoxide and absorbance at 365 nm was attained by the present procedure as is evident from the standard curve obtained with PCOOH (Fig. 1). No significant deviation from linearity was seen as judged by linear least squares analysis. Although we normally measured less than 30 nmoles of hydroperoxide, we observed that proportionality was maintained up to 70 nmoles using a wide range of hydroperoxides. This is in contrast to iodometric assays of hydroperoxides based on starch iodine complex formation (12,13). These assays are about as sensitive as the present method when using small volumes (13) but depart significantly from linearity above 15 nmoles of hydroperoxide. They suffer further disadvantages due to possible variability in the quality of the soluble starch. In the present assay, the molar absorptivity at 365 nm (see inset, Fig. 1) for I_3^- derived from enzymatically standardized H_2O_2 was $2.48 \times 10^4 \times M^{-1} \times cm^{-1}$ which is in good agreement with the value of $2.46 \times 10^4 \times M^{-1} \times cm^{-1}$ obtained by El-Saadani *et al.* (3) who directly added I_2 to the assay mixture. This shows that 18% methanol, which is present in our assay but was absent from the original, does not affect the absorbance of the reaction product. While testing the tolerance of the assay system to methanol, using either H_2O_2 or PCOOH, we found no effect of methanol up to 20%, but decreased color development was seen at 30% methanol. When freshly prepared with peroxide-free Triton X-100, our reagent gives a blank absorbance of about 0.01, but the method can still be used with blanks up to 0.2, as we noted when using Triton X-100 that had not been especially purified.

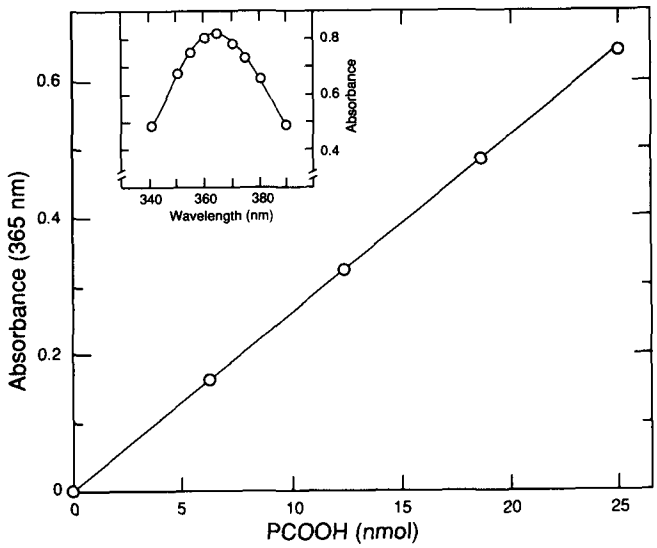

FIG. 1. Standard curve for phosphatidylcholine hydroperoxide (PCOOH) and absorbance maximum for triiodide ions. The mean absorbances from duplicate samples of PCOOH obtained as described in Materials and Methods are plotted vs. nmoles of PCOOH added. The maximum range of the duplicates was less than 1% of the mean at each point. The curve of absorbance vs. wavelength (inset) was obtained from a sample containing 36 nmoles of H_2O_2 using a single-beam spectrophotometer calibrated to within 0.5 nm with a holmium oxide filter.

The kinetics of I_3^- formation from representative hydroperoxides of various types are shown in Figure 2. PCOOH and LinOOH showed similar reactivities with both essentially complete within the recommended 30 min incubation time at 50°C. Hydrogen peroxide went to completion in all samples except for the two shortest incubation times at 30°C. CuOOH was the least reactive, reaching 87% of its maximum value within 30 min at 50°C. Thus, when applying the method to hydroperoxides of un-

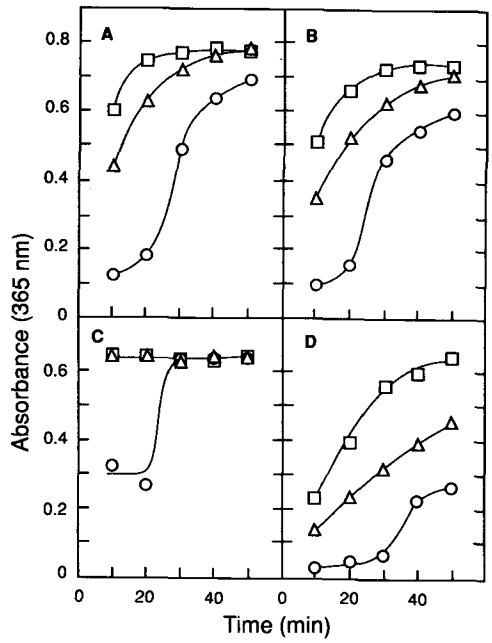

FIG. 2. Effect of temperature on color development for various hydroperoxides. Reactions were set up at 5 times the usual volume, containing, A, 32 μM phosphatidylcholine hydroperoxide; B, 30 μM linoleic acid hydroperoxide; C, 26 μM H_2O_2; D, 25 μM cumene hydroperoxide, and incubated in a water bath at 30°C (○), 40°C (△) and 50°C (□). Timing began with the addition of color reagent, and 1.0-mL samples were removed at various times and held on ice for no longer than 3 min when all samples for a particular time point were read against water. The absorbance of the reagent blank (*ca.* 0.01) did not change with time at any of the temperatures.

known reactivity, it is important to ensure that an appropriate incubation time is chosen.

El-Sadaani *et al.* (3) had observed that interference from molecular oxygen in the assay was minimal; blank values measured immediately after mixing were the same as those measured after 30 min at ambient temperature. However, these authors recommended that the 30-min color development proceed in the dark. We have made no attempt to exclude normal room lighting or oxygen and have found the method to be very forgiving in this respect. Table 1 shows the stability of absorbance when a series of assay tubes was processed with the recommended 30-min incubation time at 50°C and then allowed to stand on the bench top for up to 15 h before measurement. Samples included PC, PCOOH, Lin, LinOOH and a reagent blank, all with and without EDTA/BHT at the levels originally recommended (3). In each sample, the absorbance values were exceptionally stable, indicating that autoxidation of I^- does not occur, even without exclusion of air, nor was there any significant peroxide formation from the unoxidized lipids or from Triton X-100 in the color reagent. Although not shown, $FeCl_3$ (5 μM) in the absence of EDTA/BHT had no effect on the assay of the samples listed in Table 1. $CuCl_2$ (5 μM) caused an increase in the absorbance of the reagent blank equivalent to 3 nmoles of hydroperoxide, but subtraction of this blank value brought the assay of added lipid hydroperoxides into line.

TABLE 1

Stability of Absorbance of Samples at 365 nm with Time, Plus and Minus EDTA/BHT[a]

Lipid[b]	EDTA/BHT	Time after 50°C incubation (h) 0	1.5	3	15
		Hydroperoxide (nmoles)			
None	+	0.4	0.4	0.6	0.6
	–	0.3	0.4	0.5	0.5
PCOOH	+	18.4	18.5	18.3	16.7
	–	18.9	18.3	19.0	18.8
PC	+	0.7	0.7	0.7	0.6
	–	0.7	0.8	1.0	1.0
LinOOH	+	22.4	22.4	22.2	23.3
	–	22.9	24.3	24.8	24.3
Lin	+	0.5	0.4	0.4	0.6
	–	0.5	0.5	0.5	0.9

[a]Replicate samples were prepared as described in Materials and Methods with or without 20 μM butylated hydroxytoluene (BHT) added in methanol and 24 μM ethylenediaminetetraacetic acid (EDTA) added in water. After 30 min at 50°C, samples were read at once against a water blank, or left at room temperature for the specified time before reading, with the absorbance at 365 nm being converted to nmoles of hydroperoxide using the molar absorptivity given in the text. PCOOH, phosphatidylcholine hydroperoxide; PC, phosphatidylcholine; LinOOH, linoleic acid hydroperoxide; Lin, linoleic acid.

[b]Approximately 20 nmoles of each lipid was added per tube.

Although we normally add KI to the color reagent just before use, reagent stored for 48 h at room temperature and unprotected from light gave indistinguishable results from those obtained with fresh reagent.

Table 2 shows that the iodometric method described here gives uniform results with various hydroperoxides. Five different preparations of hydroperoxide were assayed iodometrically, and the same preparations were assayed enzymatically using both GPX and PHGPX. Good agreement among the three methods was obtained for the apparent concentrations calculated from the raw data, representing a range of sample sizes and dilutions. As expected from the known specificity of the two peroxidases (8), PCOOH could not be detected with GPX but was detectable with PHGPX. A photooxidized crude extract of retinal lipids, containing a mixture of hydroperoxides, was also unreactive with GPX but reacted with PHGPX.

We have found the present assay useful for relatively pure samples of various hydroperoxides, but less useful for direct measurement of endogenous hydroperoxides in crude tissue extracts. When either rat brain or rat retina was extracted, partitioned into dichloromethane and redissolved in methanol, essentially as described by van Kuijk *et al.* (4), no positive iodometric assay could be obtained on these samples. Furthermore, the presence of such tissue extracts prevented the normal development of color from added H_2O_2 or CuOOH. However, when similar samples were first photooxidized in the presence of rose bengal, no interference with the reaction was seen (Table 2). The interfering material has not been identified, but vitamin E is present in crude lipid extracts and known to interfere with another oxidation/reduction assay for

TABLE 2

Comparison of the Iodometric Method with Enzymatic Methods for Estimating Various Hydroperoxides[a]

	Iodometric method[b]	Enzymatic methods[c]	
		GPX	PHGPX
	Calculated concentration of hydroperoxide (mM)		
H_2O_2	1.82 ± 0.01	1.82 ± 0.01	1.83 ± 0.01
CuOOH	2.09 ± 0.06	2.22 ± 0.06	2.25 ± 0.09
LinOOH	1.73 ± 0.01	1.67 ± 0.04	1.81 ± 0.01
PCOOH	1.70 ± 0.08	0	1.50 ± 0.05
Retinal lipid hydroperoxides	0.90 ± 0.006	0	0.83 ± 0.09

[a]Each number was calculated from the mean of two determinations plus and minus the observed range. H_2O_2 and cumene hydroperoxide (CuOOH) were commercial samples. PCOOH was made by dye-catalyzed photooxidation as described in Materials and Methods. LinOOH and retinal lipid hydroperoxides were made by subjecting linoleic acid, and a lipid-rich extract of rat retina, respectively, to similar photooxidation procedures. See Table 1 for other abbreviations.

[b]Samples were analyzed iodometrically in duplicate as described in this paper. The concentration of H_2O_2, as evaluated enzymatically, served as the standard value for the iodometric assays.

[c]Samples were analyzed enzymatically by coupling reduction of the hydroperoxides by glutathione catalyzed by commercial glutathione peroxidase from bovine erythrocytes (GPX) or by phospholipid hydroperoxide glutathione peroxidase (PHGPX; Ref. 8) to the oxidation of NADPH by oxidized glutathione catalyzed by yeast glutathione reductase. For further details of the enzymatic procedure, see Materials and Methods section.

hydroperoxides (14). Added α-tocopherol inhibited our present assay stoichiometrically, causing a mole-for-mole decrease in the absorbance expected from added hydroperoxide standards. Furthermore, adding increasing amounts of H_2O_2 to a reaction mixture containing an excess of α-tocopherol caused a proportional decrease in absorbance at 292 nm and also in fluorescence emitted at 340 nm after excitation at 295 nm, suggesting alteration of the conjugated ring of α-tocopherol. It may be that the conjugated ring of vitamin E in fresh tissue extracts reacts with the newly-produced I_2 under our assay conditions, making it unavailable to form the I_3^- chromophore. Unsaturated fatty acid side chains are another potential target for iodination. However, we observed no interference with the estimation of 30 nmoles of H_2O_2, LinOOH or PCOOH, from adding 400 nmoles of Lin or 200 nmoles of PC, quantities near the solubility limit for our standard reaction mixture. Additional work will be required to adapt this method for general application to hydroperoxide measurements in crude tissue extracts.

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A Microprecipitation Technique Suitable for Measuring α-Lipoprotein Cholesterol

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A semi-automated method has been developed for determining α-lipoprotein cholesterol values. Precipitation of apolipoprotein B containing lipoproteins takes place in wells of microtiter plates after 100 μL of serum are mixed with 20 μL of a heparin/$MnCl_2$ solution. A Beckman (Fullerton, CA) Biomek 1000 work station is used to transfer sera, supernatants and reagents between tubes and microtiter plates. Supernatant cholesterol is determined enzymatically, and absorbances are read at 490 nm using a Molecular Devices Corporation (Palo Alto, CA) plate reader. Values obtained on both fresh and frozen serum samples agreed with corresponding data obtained at the Centers for Disease Control (CDC; Atlanta, GA). For the fresh samples, the average bias was 2.87%. The within-run coefficients of variations were between 2.2 and 0.6% for the data obtained on CDC frozen control pools. The results indicate that the semi-automated method is suitable for obtaining accurate and precise data for α-lipoprotein cholesterol. The method lends itself to the analysis of large numbers of samples and is particularly suited for the study of lipoproteins of small mammals.

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With advancements in the design of plate readers, microtiter plates can be used to measure concentrations of both total plasma cholesterol and triacylglycerol (1–3). Utilizing the Biomek 1000 (Beckman, Fullerton, CA) work station in conjunction with the V_{max} Microplate Reader (Palo Alto, CA), it was possible to use microtiter plates to precipitate apolipoprotein (apo) B containing lipoproteins and to measure α-lipoprotein cholesterol. The general term "α-lipoprotein cholesterol" is used throughout this paper instead of high density lipoprotein (HDL) cholesterol because the procedures were developed to study the lipoproteins of a variety of mammals in which α-lipoproteins can be highly polydisperse, being present in both the low density lipoprotein (LDL) and HDL ultracentrifugal classes (4–6).

A variety of polyanions in association with divalent cations can be used to precipitate apo B-containing lipoproteins (7) that, in mammalian plasma, can contain either apo B100 (molecular weight, 545,000) or apo B48 (molecular weight, 260,000) (8). On the surface of the triacylglycerol-rich lipoproteins, either one of the two forms of apo B is present, and apo B100 is on the surface of β-migrating LDL. Heparin/$MnCl_2$ was selected as the precipitant in this method because in studies of various mammalian species (9), including humans (7,9,10), it has been shown to be selective in precipitating apo B-containing lipoproteins.

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Abbreviations: apo, apolipoprotein; CDC, Centers for Disease Control; HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); PIPES, piperazine-*N,N'-bis*(2-ethanesulfonic acid).

The present paper describes how α-lipoprotein cholesterol values can be determined after 20 μL of a heparin/$MnCl_2$ solution and 100 μL of serum are mixed in wells of microtiter plates. Enzymatic reagents are used to measure α-lipoprotein cholesterol with analyses done on 20 μL of supernatant. The absorbance of each of the 96 wells of the microtiter plates is read within 5 s. Data on fresh and frozen serum samples compared with values obtained at the Centers for Disease Control (CDC; Atlanta, GA) indicate that the semi-automated method is both accurate and precise.

MATERIALS AND METHODS

Frozen serum samples. Frozen samples consisting of one of three pools, A, B and C, were obtained from the CDC Lipid Standardization Laboratory (Atlanta, GA). On separate days, six vials of a given pool were analyzed.

Fresh serum samples. Fasting serum samples were obtained from ten individuals at the Medical College of Virginia (Richmond, VA) and sent refrigerated overnight to UCLA (Los Angeles, CA) where analyses were done the day after the blood was drawn.

Enzymatic cholesterol reagent. The reagent, 50 mM piperazine-*N,N'-bis*(2-ethanesulfonic acid) (PIPES) buffer (Sigma; St. Louis, MO), pH 6.9, was prepared as described by Warnick (11) with the following modifications. Lyophilized enzymes (International Bioproducts, Streamwood, IL) were dissolved in 3 M NaCl and stored at –20°C. Enzymes were added on the day of the assay to give the following concentrations: horseradish peroxidase, 10 U/mL; cholesterol esterase 0.2 U/mL; and cholesterol oxidase, 0.2 U/mL. Phenol was replaced by 1.5 mM sodium 2-hydroxy-3,5-dichlorobenzenesulfonic acid (Research Organic, Inc., Cleveland, OH).

Initial set-up of the Biomek 1000. Because the Biomek 1000 moves rapidly during programmed maneuvers, 96-hole plastic racks (Bremshield Corp., Van Nuys, CA) were specially designed both to keep the racks in position on the movable tray and to maintain 1 mL polystyrene tubes (DBM Scientific, Valencia, CA) tightly in a vertical position. The spacing of holes in the rack was identical to the spacing of wells in a standard 96-well plate. For all of the programmed maneuvers, the MP 200 tool with eight tips was used to aspirate, to dispense and to mix the sera, solutions and supernatants.

Heparin/$MnCl_2$ precipitation protocol. The micromethod for precipitating apo B containing lipoproteins was based on modification (12) of the procedure of Burstein *et al.* (7). Before each run, serum sufficient for the analysis was transferred with Pasteur pipettes into 1 mL tubes arranged in columns in a Bremshield rack. The remaining steps were done with the Biomek. First, preselected wells of 96 round-bottom well plates (Costar, Cam-

bridge, MA) were filled, eight at a time, with 20 μL of a solution of sodium heparin (1099 U/mL, Liquaemin Sodium; Organon, W. Orange, NJ) and 0.275 M $MnCl_2$. Then, from each tube 100 μL of serum was transferred to a well containing the heparin/$MnCl_2$ solution. This step was done by transferring sera from a column of eight tubes at a time to the corresponding column of eight wells. To mix the sample with the precipitating reagent, plates were vibrated for 15 s in the V_{max} Microplate Reader and then incubated for 20 min at 4°C. Using a plate holder that fits the TH-4 rotor, plates were spun at 4°C and 1500 × *g* for 20 min in a Beckman TJ-6 centrifuge.

Measurement of α-lipoprotein cholesterol. The Biomek 1000 was programmed to aspirate 20 μL of supernatant from wells, eight in a column, and to dispense this volume into a corresponding column of tubes in a Bremshield rack. Next, 600 μL of enzymatic reagent were dispensed manually with an 8-tipped Titertek Multistepper (ICN Flow Labs., Costa Mesa, CA). The Bremshield rack containing the tubes was incubated in a 37°C water bath for 15 min. Then, the rack was returned to the Biomek 1000, which first mixed the solution in each tube by aspirating and dispensing twice 180 μL and then transferred three, 175-μL aliquots into wells of a 96 flat-bottom well plate (Costar). Routine monitoring of these plates at 490 nm has revealed that the optical path lengths of the wells are quite uniform. However, the plates were not used if visible scratches were observed. Saline blanks were prepared by adding 100 μL of solution (0.19 M NaCl) to 20 μL of heparin/$MnCl_2$ solution. A 20-μL aliquot of a 50-mg/dL Sigma calibrator without any precipitating reagent was included in each run as a standard. Using this standard, the concentrations were calculated on an IBM PS/2 computer using software from Molecular Devices after plates were read at 490 nm in the V_{max} Microplate Reader. A dilution correction of 1.20, resulting from precipitant addition, was incorporated into the calculations. The values reported in the Results section in Tables 1 and 2 for each sample were determined from the means of readings of three wells.

RESULTS

High blank values have previously been reported when enzymatic reagents prepared with phosphate buffer were used to analyze heparin/Mn^{2+} supernatants (13,14). However, with the enzymatic reagent prepared with PIPES buffer, difference in absorbance at 490 nm between the saline/heparin/Mn^{2+} blank and the reagent blank in this study was never greater than 0.004. This difference amounted to a correction that varied between 0.7 and 0.2 mg/dL for the various runs. Analyses of the CDC pools indicated that the assay was linear for absorbances between 0.160 and 0.450. Each value in Table 1 consists of the mean of three separate measurements obtained using one of the vials containing a specific control pool. The coefficient of variation for any one of these sets of three measurements was never greater than 2.39%. Moreover, the data for the mean of the means for these sets of measurements resulted in coefficients of variation being less than 2.2%. Comparing the data to the CDC target values, the bias was positive and ranged between 1.24 and 6.34%. All of the values fell within the range limits, ±10% from the target value, as required by the CDC (15). The upper limits for standard deviations required by the CDC are 2.50 for pool A, 3.00 for pool B and 3.50 for pool C.

Table 2 lists the cholesterol values when fresh serum was used to obtain heparin/Mn^{2+} supernatants at our laboratory and at the CDC. The set of samples sent to the CDC were frozen at −70°C, and the precipitation and analyses were done a week later. The data obtained in both laboratories agreed quite well. The highest bias among the ten samples was 7.59%. Data were also obtained on a diluted control pool. All of the data obtained with the semi-automated method were well within the CDC limit of ±10% from their observed value (15).

DISCUSSION

Throughout the Lipid Research Clinic studies, heparin/$MnCl_2$ was the precipitating reagent used to

TABLE 1

α-Lipoprotein Cholesterol (mg/dL)[a] Values Obtained on CDC Frozen Pool Samples

	Run 1			Run 2		
CDC pools	A	B	C	A	B	C
target values	29.9	50.3	80.0	29.9	50.3	80.0
	30.0 ± 0.11	53.2 ± 0.00	82.7 ± 0.19	30.7 ± 0.10	53.9 ± 0.70	82.7 ± 0.11
	30.7 ± 0.11	53.7 ± 0.37	86.1 ± 0.00	30.1 ± 0.18	52.0 ± 0.11	81.3 ± 0.28
	30.1 ± 0.44	53.3 ± 0.27	87.7 ± 0.48	30.2 ± 0.10	54.2 ± 0.00	81.5 ± 0.38
	30.5 ± 0.19	52.7 ± 0.18	84.4 ± 0.29	30.7 ± 0.10	51.7 ± 0.32	82.5 ± 0.66
	29.8 ± 0.72	53.3 ± 0.51	83.7 ± 0.58	30.9 ± 0.10	53.9 ± 0.66	83.0 ± 0.18
	30.5 ± 0.11	53.5 ± 0.20	85.8 ± 0.48	31.1 ± 0.10	53.9 ± 0.38	82.7 ± 0.11
Mean ± SD	30.27 ± 0.35	53.28 ± 0.34	85.07 ± 1.81	30.62 ± 0.39	53.27 ± 1.11	82.28 ± 0.71
C.V. (%)	1.16	0.64	2.13	1.27	2.08	0.86
Bias (%)	1.24	5.92	6.34	2.41	5.90	2.85

[a]Values are the means ± SD of readings of three wells. CDC, Centers for Disease Control (Atlanta, GA).
[b]C.V., coefficient of variation.

TABLE 2

α-Lipoprotein Cholesterol (mg/dL) Values Obtained on Fresh Serum Samples

Sample no.	Semi-Auto[a]	CDC data[b]	CDC mean	Bias (%)[c]
1	53.54 ± 0.59	50.3 and 50.7	50.50	6.02
2	35.13 ± 0.18	35.9 and 35.9	35.90	−2.14
3	62.83 ± 0.18	60.8 and 60.5	60.65	3.59
4	62.89 ± 0.38	61.9 and 61.8	61.85	1.68
5	91.33 ± 0.28	87.2 and 87.4	87.30	4.62
6	52.01 ± 0.18	50.0 and 49.9	49.95	4.12
7	69.07 ± 0.18	64.1 and 64.3	64.20	7.59
8	42.59 ± 0.74	41.6 and 41.9	41.75	2.01
9	51.21 ± 0.46	50.3 and 51.1	50.70	1.01
10	59.90 ± 0.32	60.0 and 59.6	59.80	0.17
Serum control	41.28 ± 0.27		41.70[d]	−1.06

[a]Means ± SD obtained from readings of three wells. Semi-Auto, semi-automated.
[b]The Centers for Disease Control (CDC) cholesterol values were obtained using the Abell–Kendall reference method (16).
[c]Percent bias (%) of the semi-automated values from the CDC mean.
[d]The CDC quality control pool was diluted fourfold.

determine the distribution of cholesterol among lipoprotein classes (12). A 3-mL volume was used for precipitation in these studies (12). Using microtiter plates, we were able to reduce this volume to 100 μL. Although sample volume is usually not a concern when dealing with human plasmas, such protocols are indispensable when characterizing the plasma lipids of a small mammal. Increasingly, microtiter plates are being used to determine plasma lipid concentrations because of the savings in time and reagents. All of the 96 wells can be read in 5 s, and the data can be stored in a personal computer and processed. The reagent volume is reduced almost two- to fourfold. Further savings can be attained by preparing the enzymatic reagents instead of using commercial kits.

Incomplete precipitation is a problem common to precipitating procedures when analyzing hypertriglyceridemic samples. In our laboratory, such samples are identified by first measuring plasma triacylglycerol concentrations. If the concentration is greater than 400 mg/dL, precipitation only is done on the ultracentrifugally isolated 1.006 g/mL bottom fraction. If the plasma is observed to be turbid or if the triacylglycerol concentration is between 150 and 400 mg/dL, the plasma is diluted twofold before precipitation. In lieu of these steps, careful inspection of the 96 well plates following their centrifugation has enabled us to identify any contaminated supernatants.

In conclusion, we describe here how 100 μL of plasma or serum can be used for a rapid and precise determination of α-lipoprotein cholesterol using heparin/Mn^{2+} as the precipitant. With the Biomek 1000, the initial loading of serum in all 96 wells can be done in less than eight minutes. The combination of the work station with the rapid reading capability of the plate reader provides the ability to determine over 380 α-lipoprotein cholesterol values in approximately four hours. Based on the values reported here, it is possible with this system to obtain data with a precision and accuracy suitable for CDC standardization. Finally, preparing the enzymatic reagent with PIPES buffer does not give rise to any of the problems (13,14) encountered when phosphate buffered reagents are used to analyze heparin/Mn^{2+} supernatants.

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ARTICLE

Trp89 in the Lid of *Humicola lanuginosa* Lipase Is Important for Efficient Hydrolysis of Tributyrin

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To determine whether Trp89 located in the lid of the lipase (EC 3.1.1.3) from *Humicola lanuginosa* is important for the catalytic property of the enzyme, site-directed mutagenesis at Trp89 was carried out. The kinetic properties of wild type and mutated enzymes were studied with tributyrin as substrate. Lipase variants in which Trp89 was changed to Phe, Leu, Gly or Glu all showed less than 14% of the activity compared to that of the wild type lipase. The Trp89Glu mutant was the least active with only 1% of the activity seen with the wild type enzyme. All Trp mutants had the same binding affinity to the tributyrin substrate interface as did the wild type enzyme. Wild type lipase showed saturation kinetics against tributyrin when activities were measured with mixed emulsions containing different proportions of tributyrin and the nonionic alkyl polyoxyethylene ether surfactant, Triton DF-16. Wild type enzyme showed a $V_{max} = 6000 \pm 300$ mmol · min^{-1} · g^{-1} and an apparent $K_m = 16 \pm 2\%$ (vol/vol) for tributyrin in Triton DF-16, while the mutants did not show saturation kinetics in an identical assay. The apparent K_m for tributyrin in Triton DF-16 was increased as the result of replacing Trp89 with other residues (Phe, Leu, Gly or Glu). The activities of all mutants were more sensitive to the presence of Triton DF-16 in the tributyrin substrate than was wild type lipase. The activity of the Trp89Glu mutant was decreased to 50% in the presence of 2 vol% Triton DF-16 compared to the activity seen with pure tributyrin as substrate. Wild type lipase and all mutants except Trp89Glu had the same affinity for the substrate interface formed by 15.6 vol% tributyrin in Triton DF-16. The Trp89Glu mutant showed a lower affinity than all the other lipase variants for the interface of 15.6 vol% tributyrin in Triton DF-16. The study showed that Trp89 located in the lid of *H. lanuginosa* lipase is important for the efficient hydrolysis of tributyrin and that this residue plays a role in the catalytic steps after adsorption of the lipase to the substrate interface.

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Triglyceride lipases (EC 3.1.1.3) are enzymes that catalyze the hydrolytic digestion of neutral lipids. Triglyceride lipases are activated at the lipid/water interface formed by their water-insoluble substrates (1–3). The activation process was early suggested to be associated with a conformational change of the enzyme (2,4). The three-dimensional structures of the lipases from *Rhizomucor miehei* (5,6), human pancreas (7) and *Geotrichum candidum* (8,9) all show an active site that is covered by one or more surface loops. The three-dimensional structures of *R. miehei* lipase-inhibitor complexes (10,11) have served as a model for the interfacial activation of that lipase. The α-helical lid (surface loop), which covers the active site of the free lipase, was displaced in the lipase-inhibitor complexes, providing access for the substrate to the active site of the enzyme. The activation of human pancreatic lipase (12), *G. candidum* (8,9) and *Candida rugosa* (13) lipases involves structural rearrangements of more than one surface loop. The amphiphilicity of the lid covering the active center of human lipoprotein lipase has been shown to be important for substrate specificity (14). A specific single-site modification of the lids of *R. miehei* and *Humicola lanuginosa* lipases has been shown to alter the activity and the specificity of the enzymes (15,16).

The two homologous lipases from the filamentous fungi *R. miehei* and *H. lanuginosa* have similar active sites (16), and the amino acid sequences of the lids covering the active site show high homology (17). The present investigation is focused on the conserved Trp residue in the lid of the two lipases (17). Chemical modification of Trp residues in the lipases from *H. lanuginosa* (18) and *Rhizopus delemar* (19) has shown that some Trp residues are important for enzyme activity, but the exact modification sites were not determined. Analyses of the three-dimensional structures of the *R. miehei* lipase (5,6) and lipase-inhibitor complexes (10,11) have shown that in the closed conformer the Trp residue located in the lid blocks the position at the active site where the inhibitor is bound in the open-lid structure. In the open-lid structure, the Trp residue covers part of the entrance to the active site and the Trp residue takes part in the formation of a large exposed hydrophobic surface upon activation (10,11).

In the present paper we demonstrate the importance of Trp89 in the lid of *H. lanuginosa* lipase to obtain a fully active enzyme for the hydrolysis of tributyrin. This was shown by site-directed mutagenesis and by following enzyme kinetics. The possible functional role of Trp89 in the lid is discussed.

MATERIALS AND METHODS

Chemicals. Tributyrin, Triton DF-16 (nonionic ethoxylate polyoxyethylene ether of linear primary alcohols), 3-(*N*-morpholino)propanesulfonic acid (MOPS), tris(hydroxymethyl)aminomethane (TRIS)-acetate and gum arabic were purchased from Sigma (St. Louis, MO). Calcium chloride and ammonium acetate were obtained from

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Abbreviations: DEAE, diethyl aminoethyl; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OD_{280}, optical density measured at 280 nm; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TRIS-acetate, tris(hydroxymethyl)aminomethane acetate.

Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

Cloning and expression of the H. lanuginosa *lipase.* The gene from *H. lanuginosa* encoding the triglyceride lipase was cloned, sequenced and expressed as described by Boel and Huge-Jensen (20), and the lipase produced was isolated from the culture medium.

Site-directed mutagenesis. The method used for the mutation of Trp89 in the lipase gene has been described by Nelson and Long (21). A polymerase chain reaction (PCR) fragment containing the desired mutation was generated by using a chemically synthesized DNA-strand as one of the primers in the PCR reactions. From the PCR generated fragment, a DNA fragment carrying the mutation was isolated after cleavage with restriction enzymes and then inserted into the expression plasmid. The correct mutation was verified by DNA sequencing.

Lipase purification. About 200 mL of supernatant from the culture medium was centrifuged, and the precipitate was discarded by decantation. Ice-cold ethanol was slowly added to the supernatant which was cooled in an ice bath. The solution was centrifuged, and the precipitate was discarded. The pH of the supernatant was adjusted to 7 with NaOH, and the ionic strength was adjusted by dilution with water to be lower than that of 50 mM TRIS-acetate buffer, pH 7.

Ion exchange step I: The supernatant containing 70% ethanol was applied onto a 200 mL diethylaminoethyl (DEAE) Fast Flow Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM TRIS-acetate buffer, pH 7. The column was washed at a flow rate of 5 mL/min until the optical density measured at 280 nm (OD_{280}) of the eluate was less than 0.05. The bound protein was subsequently eluted with a linear NaCl gradient (five column volumes) up to 0.5 M NaCl in 50 mM TRIS-acetate buffer, pH 7. Lipase activity was eluted between 0.1 and 0.15 M NaCl.

Hydrophobic interaction chromatography step: The fractions containing lipase activity were pooled, and ammonium acetate was added to a final concentration of 0.8 M. The solution was applied on a 100-mL Toyopearl™ Butyl-650C column (TosoHaas, Montgomeryville, PA). The column was equilibrated with 0.8 M ammonium acetate at a flow rate of 5 mL/min and was washed until OD_{280} was below 0.05. The bound activity was eluted with distilled water. The fractions with lipase activity were pooled, and the conductivity of the solution was adjusted by dilution with water to be less than that of 50 mM TRIS-acetate buffer, pH 7.

Ion exchange step II: The lipase solution was applied onto a 30-mL Q-Sepharose High Performance column (Pharmacia) equilibrated with 50 mM TRIS-acetate buffer, pH 7. The bound activity was eluted with a linear salt gradient up to 1 M NaCl at a flow rate of 1 mL/min.

No contaminants could be detected in the purified lipase after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Commassie Blue staining.

Protein quantitation. Protein concentrations were determined spectrophotometrically at 280 nm (wild type *H. lanuginosa* lipase, $\varepsilon = 4.3 \times 10^4\ M^{-1}cm^{-}$; Trp mutants, $\varepsilon = 3.6 \times 10^4\ M^{-1}cm^{-1}$).

Enzyme activity assay. Tributyrin (0.5 mmol) was added to a water solution (5 mL) of gum arabic [5% (wt/vol)] and calcium chloride (0.2 M), pH 7.5. The mixture was emulsified by sonication for one minute. The enzyme was dissolved in 10 mM MOPS buffer, pH 7.5. The reaction was started by the addition of enzyme solution to the stirred, thermostatted (25°C) substrate solution (1.5 mL). The pH was maintained automatically with sodium hydroxide (10–100 mM), using a Radiometer pH-stat equipped with an ABU91 autoburette (1 mL) connected to a VIT90 videotitrator. The reactions were run for 5 min under nitrogen. In experiments with Triton DF-16, tributyrin and Triton DF-16 were mixed before being added to the water solution and then sonicated. The total volume of tributyrin plus Triton DF-16 was kept constant at 32 mL/L of water (for 100% tributyrin equal to 0.1 M).

RESULTS

Hydrolysis of emulsified tributyrin. The enzyme variants in which Trp89 had been changed to Phe, Leu, Gly or Glu, all showed less than 14% of the activity obtained with the wild type enzyme in a standard tributyrin assay, pH 7.5 (Table 1). The same activities were obtained with 0.1 or 0.2 M tributyrin. The Trp89Glu mutated enzyme was the least active, expressing only 1% of the activity of wild type enzyme. A different activity-pH dependence was seen for the Trp89Glu mutant compared to those of the Trp89Gly mutant and the wild type enzyme (Fig. 1).

The apparent interfacial $K_{m,i}$ values (3,22) of the wild type and the Trp89 mutants of the lipase were the same, $K_{m,i} = 12\ m^2/L$ (0.017 M) for the interface obtained with pure tributyrin as a substrate (Fig. 2). The tributyrin droplet radius in the emulsion was estimated by microscopy to be r = 1.3 μm (distribution, r = 0.7–2 μm).

Hydrolysis of mixed emulsions of tributyrin and Triton DF-16. Wild type lipase showed saturation kinetics when the tributyrin concentraton was increased from 0 to 100% in Triton DF-16. The total volume of tributyrin plus Triton DF-16 was kept constant at 32 mL/L of water (for 100% tributyrin this amounted to 0.1 M, or in substrate surface to 68 m^2/L emulsion). Wild type enzyme showed a $V_{max} = 6000 \pm 300\ mmol \cdot min^{-1} \cdot g^{-1}$ and an apparent $K_m = 16 \pm 2$ vol% tributyrin in Triton DF-16. These results were obtained by fitting experimental data directly (non-

TABLE 1

Activity of Wild Type and Trp89 Mutants of *Humicola lanuginosa* Lipase in a Standard Tributyrin Assay at pH 7.5 and 25°C

Enzyme	Activity[a] (mmol/min · g)
Wild type	5400 ± 200
Trp89Phe	750 ± 50
Trp89Leu	415 ± 15
Trp89Gly	295 ± 15
Trp89Glu	63 ± 5

[a]Standard deviations are based on three separate measurements.

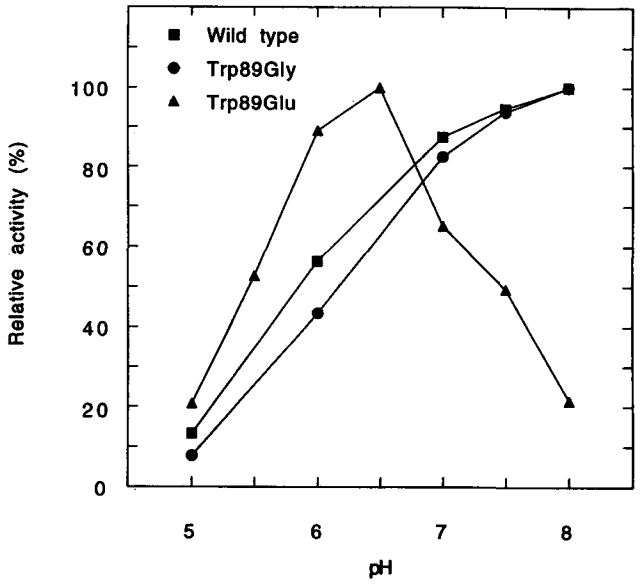

FIG. 1. Activity of wild type and Trp89 mutants of *Humicola lanuginosa* lipase as a function of pH in the hydrolysis of 0.1 M tributyrin at 25°C. A relative activity of 100% corresponds to the highest activity obtained for each enzyme variant in the pH interval 5 to 8.

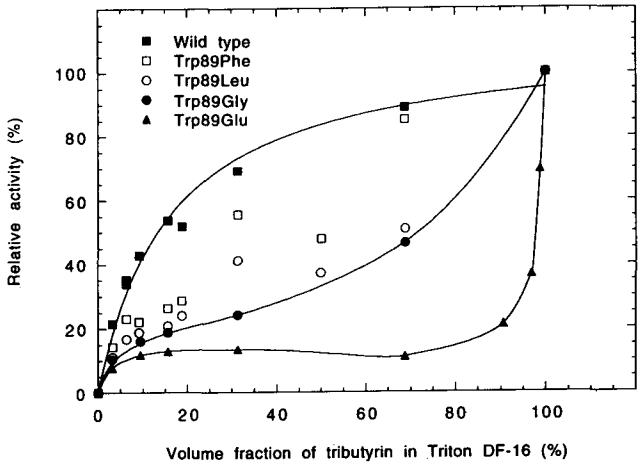

FIG. 3. Activities of wild type and Trp89 mutants of *Humicola lanuginosa* lipase in mixed emulsions of tributyrin and Triton DF-16. The volume of tributyrin plus Triton DF-16 was kept constant at 32 mL/L water in all experiments. The volume fraction 100% corresponds to 0.1 M tributyrin, and 100% activity was defined as that obtained with 0.1 M tributyrin as a substrate. Activities were measured at pH 7.5 and 25°C.

linear regression) to a Michaelis-Menten type curve (Fig. 3). The enzymes in which Trp89 had been mutated to Phe, Leu, Gly or Glu did not show saturation kinetics under the same experimental conditions and did not fit a Michaelis-Menten type curve. Instead, more complex nonlinear relationships between the activity and the volume fraction of tributyrin in Triton DF-16 were seen (Fig. 3). The activities of all mutants tested were more sensitive to the presence of Triton DF-16 in the tributyrin substrate than was the wild type enzyme. The activity of the Trp89Glu mutated lipase plateaued at a concentration of 10% tributyrin in Triton DF-16 (Fig. 3). When the tributyrin concentration was further increased (90–100%), the activity increased to 100%. Only 2 vol% Triton DF-16 was needed to reduce the activity to 50% compared to the activity obtained with pure tributyrin.

Wild type enzyme and all Trp mutants except Trp89Glu had the same apparent interfacial $K_{m,i}$ value (3,22), namely 2 m^2/L for the interface formed by 15.6% tribu-

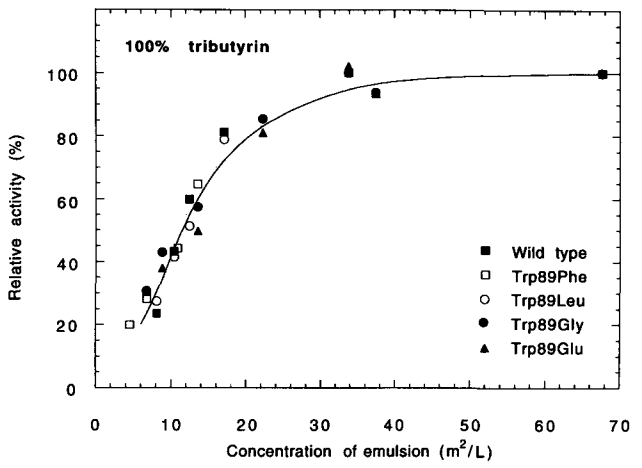

FIG. 2. Activities of wild type and Trp89 mutants of *Humicola lanuginosa* lipase as a function of the emulsion concentration (substrate surface). Experiments were performed by dilution of a stock emulsion of tributyrin (0.1 M tributyrin or in substrate surface, 68 m^2/L emulsion). The 100% activity corresponds to the activity obtained with the stock emulsion. Activities were measured at pH 7.5 and 25°C.

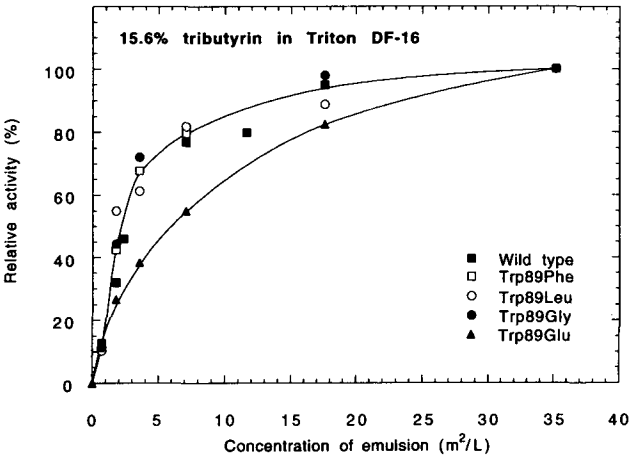

FIG. 4. Activities of wild type and Trp89 mutants of *Humicola lanuginosa* lipase as a function of the emulsion concentration (substrate surface). Experiments were performed by dilution of a stock emulsion of 15.6% tributyrin in Triton DF-16 (the total volume of tributyrin plus Triton DF-16 was 32 mL/L water or in substrate surface, 35 m^2/L emulsion). The 100% activity corresponds to the activity obtained with the stock emulsion. Activities were measured at pH 7.5 and 25°C.

tyrin in Triton DF-16 (Fig. 4). The Trp89Glu mutant had a lower affinity for the substrate than did the wild type enzyme. The apparent interfacial $K_{m,i}$ of the Trp89Glu mutant was about 7 m^2/L (Fig. 3). The droplet size obtained with the mixed emulsion of 15.6% tributyrin in Triton DF-16, r = 2.5 μm (distribution, 0.7–7 μm), was greater than the one obtained with pure tributyrin.

DISCUSSION

The ability of the lipase to hydrolyze emulsified tributyrin, either pure or mixed with Triton DF-16, was considerably decreased when Trp89 was changed to other residues (Table 1, Fig. 3). The activities of the lipase mutants were determined with an excess of substrate surface. The differences between the lipases should therefore originate from the catalytic steps occurring at the substrate interface (Fig. 5). The mutants where Trp89 had been changed to the smaller hydrophobic amino acids, Phe, Leu or Gly, showed 5–14% of the activity seen with wild type enzyme. Consequently the size and/or hydrophobicity of the residue at position 89 is important for high enzyme activity. In the Trp89Glu mutant, in which an ionizable side chain had been introduced, only 1% of the activity remained. The negatively charged residue in the lid might be unfavorable for efficient catalysis. In fact, this was seen by the different activity-pH dependence for the Trp89Glu mutant compared to those of the Trp89Gly mutant and the wild type enzyme (Fig. 1). The negatively charged Glu residue could affect the electrostatic stabilization of the position of the lid (15,16) resulting in an open conformation of the enzyme which is not optimal for catalysis. In addition, the charged Glu residue in the lid is close to the ionized fatty acid generated by the hydrolysis of the acyl-enzyme intermediate. Perhaps Glu89 prevents efficient interfacial penetration of the enzyme into the lipid phase.

Lipase bound to the substrate interface might have different conformations with different specific activities. Once the lipase has adopted its open active conformation(s), the efficiency of the catalysis depends on the ability of the enzyme to extract substrate from the lipid phase into the active site (K_m) and to subsequently convert substrate to product (V_{max}) (Fig. 5). With a standard tributyrin assay, these different steps, occurring at the substrate interface, cannot be separately investigated since the concentration of tributyrin at the interface cannot be varied. To gather further insights into the mechanism behind the considerably decreased activities seen as the result of changing Trp89 in the lid into other residues, a complementary activity assay was used in which the accessibility of tributyrin at the interface was altered by the presence of the Triton DF-16 surfactant. The results obtained show that the activities of the mutants are more sensitive to the dilution of tributyrin with Triton DF-16 than that of the wild type enzyme. Since the experiments were performed with an excess of emulsion surface, the activities reflected the steps in catalysis at the substrate interface (Fig. 5). Lipase activity has been shown to depend on the interfacial quality and/or the interfacial pressure of the substrate (23). The mixed emulsions of tributyrin and Triton DF-16 represent a complex system in which the quality of the interface and the interfacial pressure might depend on the molar ratio of the two components tributyrin and Triton DF-16. Nevertheless, the qualitative results obtained in the tributyrin/Triton DF-16 assay clearly illustrate the importance of Trp89 in the lid to obtain a functional enzyme for triglyceride digestion in the presence of Triton DF-16. The ability of a lipase to efficiently degrade lipid substrates in the presence of detergents is a desirable property of an enzyme to be used in, for instance, washing powder.

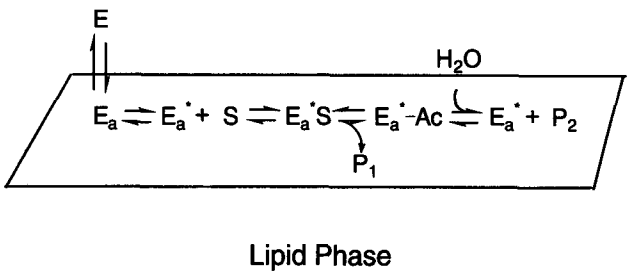

FIG. 5. Schematic model of lipolysis. E, enzyme in bulk water; E_a, enzyme adsorbed at the lipid interface; E_a^*, active enzyme adsorbed at the lipid interface; S, triglyceride substrate; E_a^*S, Michaelis-Menten complex; E_a^*-Ac, acyl-enzyme; P_1, diacylglycerol; P_2, fatty acid.

It is not clear if the steps leading to acylation or deacylation of the enzyme are rate-determining in the hydrolysis of tributyrin. Data obtained with wild type enzyme and other substrates have indicated that the acylation process is rate-determining with triglycerides as substrates (Martinelle, M., Holmquist, M., Clausen, I.G., Patkar, S., Svendsen, A., and Hult, K., manuscript in preparation). It therefore seems as if the Trp89 residue plays a role during the formation of the acyl-enzyme intermediate (Fig. 5). The ability of the lipase to efficiently form the acyl-enzyme intermediate at the interface is dependent on a number of separate steps: i) the active open-lid conformation must be formed, ii) the lipase has to penetrate the lipid interface and iii) the active site has to be saturated with substrate. From the data obtained in this investigation, we cannot determine which of these steps were affected by the mutation of Trp89 into other residues.

The lipases from *Rhizopus* species *(Rh. delemar, Rh. niveus, Rh. javanicus)* have molecular structures similar to that of the lipase from *R. miehei* (24). The Trp residue in the lid of *R. miehei* lipase corresponds to an Ala in the lipases from the *Rhizopus* species. It has been suggested that the Trp residue is unimportant for the function of the lid (22). Since other amino acid residues in the lid of the *Rhizopus* species lipases and *R. miehei* lipase are different, the importance of a particular residue in different lipase lids is difficult to estimate. Therefore, our finding that Trp89 in the *H. lanuginosa* lipase lid is important for activity should not be generalized to other lipases. The continued work toward a detailed understanding of lipase catalysis will assist in the efforts of engineering lipases with desirable properties.

ACKNOWLEDGMENTS

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Dietary Fat Composition and Age Affect Synaptosomal and Retinal Phospholipid Fatty Acid Composition in C57BL/6 Mice

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The purpose of this study was (i) to determine whether dietary fat-induced differences in neural and retinal membranes occur when dietary fat treatment is implemented in aged animals and (ii) to characterize the effect of long-term differences in dietary fat on neural and retinal membrane composition. For the first objective, young (six-week-old) and old (95-week-old) mice were randomly assigned to beef tallow (TAL) or soybean oil (SBO) diets for eight weeks. For the second objective, young (four-week-old) mice consumed either TAL or SBO diets for 99 weeks. Young and old mice challenged with a change in dietary fat for an eight-week period showed both diet and age effects on neural and retinal phospholipid fatty acid composition ($P < 0.05$). In addition, significant diet by age interactions were evident. In mice that consumed TAL and SBO diets throughout their life, only retinal phosphatidylethanolamine (PE) 18:2n-6 and neural phosphatidylserine 22:5n-6, PE 18:2n-6 and phosphatidylcholine 18:2n-6 differed between dietary treatments ($P < 0.05$). Neither the unsaturation index nor the n-6/n-3 ratio was affected by diet. Neural and retinal phospholipid fatty acid composition were responsive to changes in dietary fat even when the treatment was implemented beyond developmental or post-weanling stages. In contrast, when mice consumed TAL or SBO diets throughout their life, fewer differences in phospholipid fatty acid composition were detected, suggesting that the effect of the dietary treatment was mitigated by aging.

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Although the lipid composition of brain is less responsive to changes in dietary fat than that of peripheral tissues (1), dietary fat can affect neural membrane fatty acid composition, particularly during development when cell differentiation and rapid accretion of neural lipids occur (2). In addition, dietary fat affects the composition of brain lipids in post-weanling animals (3–5) because of turnover of phospholipid fatty acids, synaptogenesis and arborization. Notably, this effect is manifested over a relatively short period of time (four weeks) and occurs with diets that are nutritionally complete (i.e., not essential fatty acid-deficient) (4,5).

Like the brain, retinal tissue has a high concentration of long-chain polyenoic n-3 fatty acids that can be altered by dietary manipulation. Imposition of an n-3 fatty acid deficiency in a developmental model has demonstrated the sensitivity of retinal phospholipid fatty acid composition to changes in the availability of dietary n-3 fatty acids (6,7). As with neural phospholipid composition, the composition of retinal lipids in post-weanling animals responds to differences in dietary fat composition that occur in normal diets (8).

Phospholipid metabolism and fatty acid composition of neural and retinal tissues are also affected by aging (9–12). Differences in both the amount of phospholipid and phospholipid fatty acid composition occur as animals age. In general, the most notable effect is the diminution of long-chain polyenoic n-3 fatty acids, particularly 22:6n-3 (11).

Changes in the lipid composition of electrically active tissues may have important biological effects. Alterations in phospholipid fatty acid composition have been associated with changes in the activities of membrane-bound enzymes (13,14), electroretinograms (6,7) and measures of cognitive performance (15,16).

Current dietary guidelines advocate quantitative and qualitative changes in dietary fat by broad segments of the population. The impact of such changes in dietary fat on the composition of electrically active tissues in aged animals is unknown. The purpose of the present study was (i) to determine whether neural and retinal tissues of aged mice were sensitive to changes in dietary fat composition when they were initiated late in life and (ii) to determine if lifelong consumption of diets that varied in their fatty acid composition resulted in differences in fatty acid composition of phospholipids in the brain and retina of aged mice.

MATERIALS AND METHODS

Animals and diets. Female C57BL/6 mice (Jackson River Laboratories, Bar Harbor, ME) were housed in plastic cages (4–5 animals/cage) under constant environmental conditions (room temperature 22–24°C; 12-h light/dark cycle). All animals were checked daily for health problems; unhealthy animals (skin lesions, tumors, *etc.*) were excluded from the study.

Tallow (TAL) and soybean oil (SBO) diets were nutritionally adequate, purified granular mixtures containing 40% carbohydrate, 24% protein, 5% nonnutritive fiber, 2.5% vitamin mix and 5.1% mineral mix. A detailed description of diet ingredients has been published previously (17). TAL and SBO diets were isoenergetic with 20% (wt/wt) dietary fat. The fatty acid composition of the diets is described in Table 1.

Diets were designed to have adequate levels of vitamin E as an antioxidant (4400 I.U. α-tocopheryl acetate/kg diet). Levels of thiobarbituric acid reactive substances detected in diets after 48 h at room temperature were 2.1 and 3.2 μmol/kg diet for TAL and SBO, respectively (18).

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Abbreviations: MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SBO, soybean oil; SFA, saturated fatty acids; TAL, beef tallow.

TABLE 1

Fatty Acid Composition of Experimental Diets[a]

Fatty acid	(% of total fatty acids) TAL	SBO
14:0	3.9	0.3
16:0	27.8	10.8
16:1n-7	2.4	0.1
18:0	20.1	3.4
18:1n-9	35.4	20.5
18:2n-6	9.7	56.4
18:3n-3	1.1	8.8

[a]Fat was extracted from premixed diets and fatty acid profiles determined by gas chromatography. TAL, beef tallow; SBO, soybean oil.

Our previous work showed an increased mortality in mice fed 20% (wt/wt) fat diets *ad libitum* (Cinader, B., unpublished data); therefore, mice consuming TAL and SBO diets had access to food only on alternate days. All animals had *ad libitum* access to water.

Experimental design. The effect of the composition of dietary fat and age on membrane fatty acid composition was examined with a 2×2 factorial design. At either six weeks or 95 weeks of age, mice that had consumed chow diets throughout their life were randomly assigned to 20% (wt/wt) TAL or SBO diets. Diets were fed for eight weeks.

To determine if lifelong consumption of diets that differed in their fatty acid composition resulted in differences in phospholipid fatty acid composition, four-week-old animals were randomly assigned to 20% (wt/wt) TAL or SBO diets for 99 weeks. At the end of the experiments, fasted mice were decapitated, and tissues were immediately removed and frozen on dry ice. Tissues were stored at –70°C until analysis. Retinae were dissected from frozen tissue under a light microscope.

Isolation of synaptosomal plasma membrane. Synaptosomal plasma membranes were isolated according to the method of Whittaker and Barker (19). Whole brains were homogenized in 0.32 M sucrose and centrifuged. The supernatant was decanted and recentrifuged to obtain a pellet containing mitochondria, synaptosomes and myelin. This pellet was applied to a discontinuous sucrose gradient and centrifuged at 100,000 × *g* for 60 min at 1°C. Synaptosomes were removed quantitatively from the 0.8–1.2 M interface and were osmotically shocked by suspension in distilled water for 1 h. The synaptosomal fraction was reapplied to a discontinuous sucrose gradient and recentrifuged as just described. Following centrifugation, plasma membranes were collected quantitatively from the 0.8–1.2 M sucrose interface. The purity of the fraction was verified by measuring the enrichment of 5′-nucleotidase (EC 3.1.3.5) (20) and by the removal of monoamine oxidase (EC 1.4.3.4) (21).

Fatty acid analysis. Lipids were extracted from membrane preparations by the method of Folch *et al.* (22). Phospholipids were separated by thin-layer chromatography (23,24), and fatty acid methyl esters were prepared by methylation with boron trifluoride in methanol (25). Fatty acid methyl esters from phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylserines (PS) and phosphatidylinositols (PI) were analyzed by gas–liquid chromatography using flame-ionization detection (Vista 402; Varian, Palo Alto, CA). Fatty acids were identified by comparing their retention times with those of authentic standards (Nu-Chek-Prep, Elysian, MN). Results are expressed as percent of total fatty acids.

Statistical analyses. Data were analyzed using the SAS 6.03 (SAS Institute, Inc., Cary, NC) program. Factorial data were subjected to two-way analysis of variance with diet (TAL and SBO) and age (14 and 103 weeks) as main effects (26). Data comparing membrane fatty acid composition in mice that were exposed to different dietary treatments for 99 weeks were analyzed by Student's *t*-test (24). For all analyses the acceptable level of significance (Type 1 error) was 5%.

RESULTS

Effects of diet and age on synaptosomal phospholipid fatty acid composition. Both dietary fat composition and age affected synaptosomal plasma membrane phospholipid fatty acid composition. Among PE fatty acids (Fig. 1), 18:2n-6 was affected by diet alone, whereas 18:1 and 22:5n-6 were affected only by age. Diet and age each had

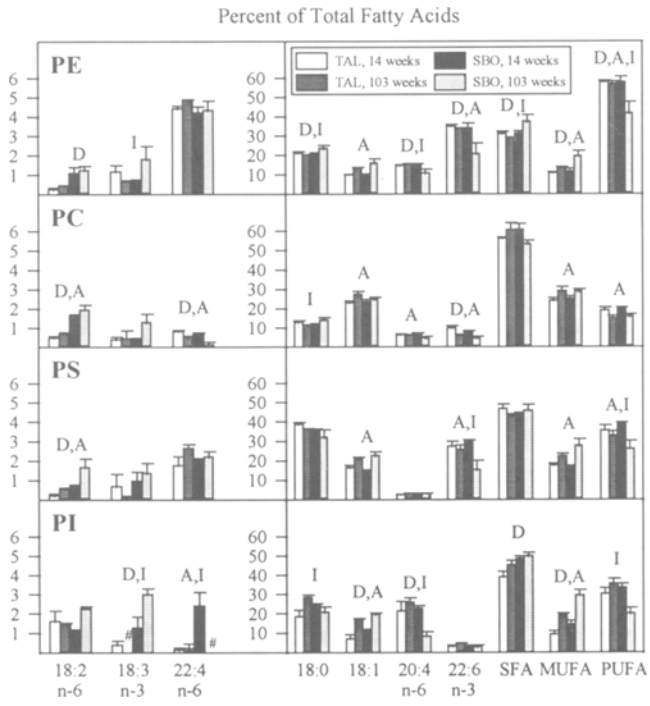

FIG. 1. Fatty acid composition of synaptosomal plasma membrane phospholipids. The inset label indicates the age at which the animals were killed. Mice were fed chow diets for six or 95 weeks, then were randomly assigned to 20% (wt/wt) beef tallow (TAL) or soybean oil (SBO) diets for eight weeks. Values are mean ± SE; n = 4–6/treatment; # indicates that the fatty acid was not detected. Letters indicate: D, significant effect of diet; A, significant effect of age; I, significant diet × age interaction ($P < 0.05$). PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

independent effects on 22:6n-3 and monounsaturated fatty acids (MUFA). There was a primary effect of diet in addition to a significant interaction term for 18:0, 20:4n-6 and saturated fatty acids (SFA). Main effects of diet and age, as well as a significant interaction term, were observed for polyunsaturated fatty acids (PUFA). The interaction of dietary fat and age was a determinant of PE 18:3n-3. The unsaturation index and the ratio of n-6/n-3 fatty acids in PE were also affected by diet and age (Table 2). There was a main effect of diet and age for both outcomes, as well as a significant interaction term for the unsaturation index.

Age was an independent factor for levels of 18:1, 20:4n-6, MUFA and PUFA in membrane phospholipids among PC fatty acids (Fig. 1). Both diet and age were independent factors for 18:2n-6, 22:4n-6 and 22:6n-3. There was a significant interaction term for 16:0 and 18:0. Age had an effect on the unsaturation index of PC fatty acids, whereas diet and age both affected the n-6/n-3 fatty acid ratio (Table 2).

Only the PS 18:2n-6 and n-6/n-3 fatty acid ratio were affected by dietary treatment (Fig. 1, Table 2). There was an independent effect of age for 18:1, 18:2n-6, 22:6n-3, MUFA, PUFA, the unsaturation index and the n-6/n-3 fatty acid ratio. A significant interaction between dietary treatment and age was observed for 16:0, 22:6n-3, PUFA, the unsaturation index and the n-6/n-3 fatty acid ratio.

Among PI fatty acids, there was a main effect of diet on 16:0, 18:1, 18:3n-3, 20:4n-6, SFA, MUFA and the n-6/n-3 ratio (Fig. 1, Table 2). Age was an independent factor for 16:0, 18:1, 20:4n-6, MUFA and the n-6/n-3 ratio. There was a significant interaction term for 16:0, 18:0, 18:3n-3, 20:4n-6, 22:4n-6, PUFA, the unsaturation index and the n-6/n-3 ratio.

When the dietary treatment was implemented in four-week-old mice and continued till senescence, dietary fat composition resulted in lesser differences in synaptosomal fatty acid composition than when the dietary treatment was implemented with 95-week-old mice for the final eight weeks of life (Fig. 2). Mice fed TAL diets throughout their life had a lower percentage of 18:2n-6 in PE, PC and PI than did mice fed SBO diets. In addition, PS 22:5n-6 was affected by diet (data not shown). Diet had no effect on either the unsaturation index or the n-6/n-3 ratio (data not shown).

Effect of diet and age on retinal phospholipid fatty acid composition. Among retinal PE fatty acids, there was an independent effect of diet on 18:2n-6, 20:4n-6, 22:6n-3, SFA and PUFA (Fig. 3). Age had an independent effect on 18:2n-6, 20:4n-6 and the n-6/n-3 fatty acid ratio (Table 2). There was a significant interaction term for 20:4n-6. Analysis of PC fatty acid composition showed a diet effect only for 18:2n-6.

When the fatty acid composition of retinal membranes from mice fed their respective diets for 99 weeks were analyzed, only PE 18:2n-6 differed between dietary fat treatments (0.24 ± 0.04% vs. 0.88 ± 0.05% of total fatty acids, TAL and SBO, respectively). Neither the unsaturation index nor the n-6/n-3 ratio was affected by diet (data not shown).

DISCUSSION

An extensive body of literature has documented the effects of aging on the lipid composition of the brain (see Ref. 27 for a review), and recent studies have examined possible mechanisms by which aging alters phospholipid fatty acid composition in brain (9,10,12) and in retina (11). Although studies on peripheral tissues have examined the combined effect of dietary fat and aging on phospholipid fatty acid composition (28,29), to our knowledge there are no data

TABLE 2

Unsaturation Index and Ratio of n-6/n-3 Fatty Acids in Synaptosomal Plasma Membrane and Retinal Phospholipids from Young and Old Mice[a]

		Unsaturation index		n-6/n-3 Ratio	
		TAL	SBO	TAL	SBO
Synaptosomal phospholipids					
PE	14 weeks	309 ± 6[bcd]	305 ± 15	0.60 ± 0.1[b,c]	0.64 ± 0.02
	103 weeks	305 ± 2	218 ± 37	0.66 ± 0.02	0.92 ± 0.13
PC	14 weeks	119 ± 6[c]	107 ± 3	0.89 ± 0.12[bc]	1.27 ± 0.04
	103 weeks	97 ± 5	91 ± 4	1.43 ± 0.15	1.66 ± 0.16
PI	14 weeks	127 ± 22[d]	148 ± 6	7.20 ± 0.69[bcd]	6.37 ± 0.90
	103 weeks	160 ± 10	102 ± 12	7.02 ± 0.66	2.29 ± 0.04
PS	14 weeks	211 ± 16[cd]	226 ± 5	0.26 ± 0.03[bcd]	0.27 ± 0.02
	103 weeks	208 ± 12	155 ± 24	0.33 ± 0.03	0.65 ± 0.14
Retinal phospholipids					
PE	14 weeks	277 ± 15	195 ± 18	0.28 ± 0.03[c]	0.47 ± 0.07
	103 weeks	256 ± 25	243 ± 12	0.48 ± 0.03	0.52 ± 0.05
PC	14 weeks	93 ± 13	108 ± 8	0.78 ± 0.16	0.61 ± 0.04
	103 weeks	89 ± 16	96 ± 21	0.79 ± 0.07	1.77 ± 0.74

[a]Mice were fed chow diets during six and 95 weeks of age, then randomly assigned to 20% (wt/wt) TAL or SBO diets for eight weeks. The label shows the age at which animals were killed. The unsaturation index was calculated from percent fatty acid data determined by gas chromatography. Values are mean ± SE, n = 4–6/treatment. Superscript letters indicate: b, significant effect of diet; c, significant effect of age; d, significant diet × age interaction ($P < 0.05$). Abbreviations as in Table 1; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

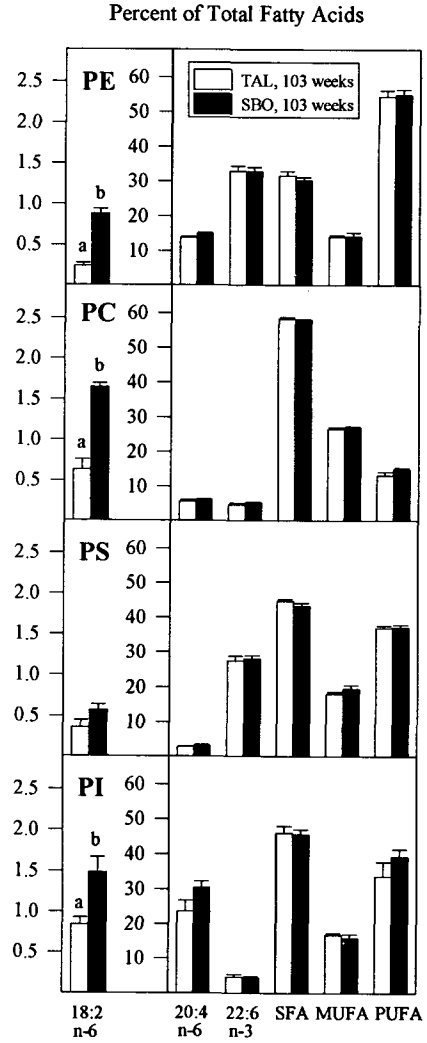

FIG. 2. Fatty acid composition of synaptosomal plasma membrane phospholipids. Mice were randomly assigned to dietary fat treatment at four weeks and consumed diets for 99 weeks. Values are mean ± SE; n = 6–8/dietary fat treatment. Values not sharing a common superscript letter are significantly different ($P < 0.05$). Abbreviations as in Figure 1.

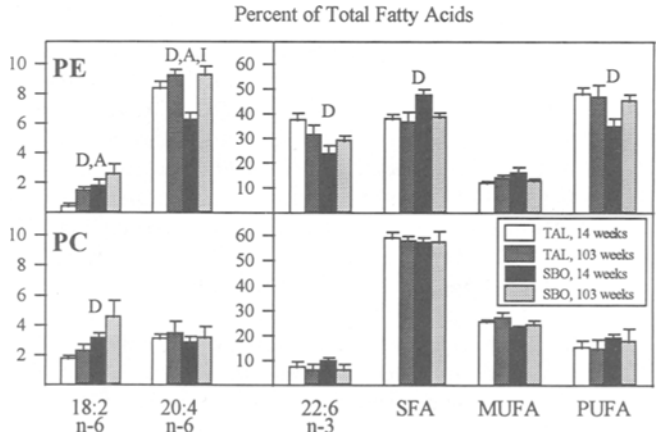

FIG. 3. Fatty acid composition of retinal PE and PC fractions. The inset label indicates the age at which the animals were killed. Mice were fed chow diets for six or 95 weeks, then were randomly assigned to 20% (wt/wt) TAL or SBO diets for eight weeks. Values are mean ± SE; n = 4–6/treatment. Letters indicate: D, significant effect of diet; A, significant effect of age; I, significant diet × age interaction ($P < 0.05$). Abbreviations as in Figure 1.

regarding the interaction of these factors on composition and function of electrically active tissues. Accordingly, the first objective of this research was to determine if diet-induced differences in neural and retinal fatty acid composition previously described in post-weanling animals also occur when the dietary treatment is implemented late in life. Mice that consumed chow diets for six or 95 weeks were subjected to a change in the amount and type of dietary fat during the final eight weeks of life. In addition to assessing the susceptibility of neural and retinal membranes in aged mice to changes in dietary fat, it was possible to determine if dietary fat and aging exerted independent and/or interactive effects on fatty acid composition of these tissues.

Our second objective was to determine if long-term consumption of diets with different fatty acid profiles resulted in differences in the composition of phospholipid fatty acids in the brain and retina of aged mice. Tissue fatty acid composition was examined in mice with a lifetime exposure (99 weeks) to different dietary fat regimens.

For both objectives, fat comprised 20% (wt/wt) of the diets, or approximately 40% of energy. This amount of fat is comparable to estimates of dietary fat as a percentage of energy in North American human populations (30,31). Because TAL and SBO were used as the source of dietary fat, the diets represent extreme points on the continuum of dietary polyunsaturated/saturated fatty acid ratios (0.21 and 4.4, TAL and SBO, respectively).

The results of the first experiment suggest that the response of electrically active tissues to a dietary fatty acid challenge is dependent upon the age of the animal when the dietary change is made. When the effect of a short-term change in dietary fat composition was examined in post-weanling (six-week-old) and old mice (95-week-old), differences due to age and dietary fat treatments were apparent in synaptosomal plasma membrane and retinal phospholipid fatty acid composition (Figs. 1 and 3). In addition to the main effects of diet and age, however, there was a dietary fat by age interaction for a number of tissue fatty acids, indicating that young and old mice did not respond in the same way to identical dietary fat treatments. Consistent with our previous observations in post-weanling rats (17), changes in the percentage of neural fatty acids in response to dietary fat treatment were most pronounced in PE and PC, whereas PI was most resistant. Differences tended to exist among the 20- and 22-carbon fatty acids.

In the second experiment, fewer differences in the percent fatty acid composition of synaptosomal plasma membrane and retinal phospholipids were evident despite lifetime exposure to qualitative differences in dietary fat (Fig. 2). These data suggest that there may be physiologic mechanisms that override the impact of prolonged differences in dietary fat. The critical exposure period is un-

known; however, data from Dyer and Greenwood (4) indicated that cardiolipin stabilized after four weeks with no change for up to 12 weeks. In contrast, differences in the ratio of n-6/n-3 22-carbon fatty acids continued to increase up to 12 weeks (5).

One factor which may have contributed to the results observed with lifetime exposure to dietary fat sources is that mice were fed on alternate days. Other studies have demonstrated changes in membrane fatty acid composition in both peripheral (32,33) and neural tissues (34) with food restriction. In addition, differences in physicochemical properties of cellular membranes have been demonstrated between *ad libitum* and restricted-fed rats; membrane viscosity of splenic lymphocytes and β-adrenergic receptor density were greater in rats with restricted food intake compared to rats fed *ad libitum* (32).

Our results cannot be directly compared with these studies because the manner in which the restricted feeding paradigm was implemented differed. Our study used an every-other-day feeding regimen because our previous work had shown an increased mortality in mice fed high fat diets on an *ad libitum* basis (Cinader, B., unpublished data). Although Pieri (32) restricted animals by feeding on alternate days, the diets contained only 6% (wt/wt) dietary fat, whereas the TAL and SBO diets used in this study contained 20% (wt/wt) dietary fat. In contrast, Tacconi *et al.* (34) food restricted their rats by diluting the energy density of diets with dietary fiber. The degree to which the differences in feeding regimens may account for differences in the results is unknown.

The effect of diet and age on the amounts of phospholipid n-3 fatty acids in electrically active tissues is of interest because of their abundance in these tissues and because they are desaturation and elongation products of 18:3n-3, an essential fatty acid. Rotstein *et al.* (11) described levels of 22:6n-3 in retina from rats 2–3 and 26–27 months old. They reported a decline with age in PC and PS 22:6n-3, as well as very long-chain polyenoic fatty acids (11). Moreover, they noted that there was no compensation by shorter chain PUFAs for this decrease, suggesting that an overall diminution in membrane fluidity occurred consequent to alterations in fatty acid composition. Neural data for young and old mice fed diets with different fatty acid composition during the final eight weeks of life revealed an age-related decrease in PE, PC and PS 22:6n-3, as well as an effect of age on the unsaturation index for each of these phospholipids. However, the effect of age on the unsaturation index was dependent on dietary fat treatment in all phospholipids except PC. These results suggest that an overall decrease in membrane fluidity occurs with aging, but the effect may be mitigated by the type of dietary fat.

The functional importance of such changes in membrane fatty acid composition has not been resolved. Developmental studies have shown that a decrease in n-3 fatty acids in retinal membrane is coincident with visual impairment in both rats and monkeys (6,7); however, a definitive link between visual function and retinal membrane composition has not been established (7,35). Studies with post-weanling animals have associated changes in dietary fat composition with a wide range of behaviors, including cognition (15,16), temperature regulation (36), pain sensitivity (36) and feeding behavior (14,17). Although differences in neural fatty acid composition have been shown to occur in a time-frame consistent with the appearance of differences in feeding behavior (17), subsequent studies failed to demonstrate causality (37). Thus, the functional significance of differences in neuronal fatty acids composition of the magnitude described is tentative as definitive mechanisms linking the biochemical and physicochemical changes with alterations in behavior have been elusive.

In summary, electrically active tissues are responsive to differences in dietary fat composition even when the treatment is implemented well beyond developmental or post-weanling stages. However, young and old mice presented with identical changes in dietary fat do not respond in the same manner due to significant diet by age interactions. Consequently, differences in fatty acid profiles previously reported in post-weanling animals may not be predictive of the magnitude or direction of change when similar dietary fat regimens are implemented with aged animals. In contrast, data from animals that consumed their respective diets from weanling to senescence indicate that electrically active tissues are more refractory to diet, suggesting that the effect of the dietary treatment is mitigated by aging.

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Central Nervous System Demyelinating Diseases and Increased Release of Cholesterol into the Urinary System of Rats

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The question of what happens to cholesterol in the adult central nervous system during its slow turnover has been addressed using rats with brain and spinal cord labeled with [4-^{14}C]cholesterol upon intracerebral injection of labeled cholesterol into rats at 10–12 days of age. At six months after injection, ^{14}C was found only in the brain and spinal cord and was slowly released *via* the rat's urine. When labeled rats were given demyelinating agents (triethyl tin chloride, hexachlorophene, sodium cyanide) and when experimental allergic encephalomyelitis was induced, a measurable increase in urinary ^{14}C label above control levels was found. It was concluded that there is a direct relationship between the experimental demyelination induced and the increased release of cholesterol metabolites into urine. The study suggests that a clinical method could be developed to determine the rate of central nervous system demyelination by measuring the amount of urinary cholesterol metabolites.

***Lipids* 29, 611–617 (1994).**

We earlier reported (1) that when [4-^{14}C]cholesterol was injected intracerebrally (IC) into immature rats, ^{14}C was excreted through the urine throughout the rats' life span. Additional evidence is now presented which indicates that six months after injection of labeled cholesterol, rats excrete ^{14}C derived from their brain and spinal cord, and it is surmised that this excretion reflects the slow turnover of cholesterol in the central nervous system (CNS), and specifically in myelin. The main purpose of the present investigation was to determine whether demyelinating agents would influence the urinary excretion of ^{14}C label derived from cholesterol. Therefore, mature labeled rats (at least six months of age) were given triethyl tin chloride [TET(Cl)], hexachlorophene (HCP) or sodium cyanide. In addition, experimental allergic encephalomyelitis (EAE) was induced in similarly prepared rats, and the ^{14}C urinary content was measured daily.

The specificity of CNS demyelination induced in young rats by oral feeding of triethyl tin sulfate makes it an appropriate tool for study of toxin-induced demyelination (2,3). Administration of small amounts of triethyl tin sulfate (5–10 mg/L) into the drinking water of rats causes a devastating loss of myelin in CNS. The demyelination occurs in the presence of severe edema and without intervention of macrophage activity (3,4).

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Abbreviations: CNS, central nervous system; EAE, experimental allergic encephalomyelitis; HCP, hexachlorophene; IC, intracerebral; TET(Cl), triethyl tin chloride.

In our studies we used TET(Cl) on the assumption that tin and not the associated ion was the critical agent. Since use of TET(Cl) has not been reported in the literature, the pathological effects of this tin salt were studied by light and electron microscopy. HCP administered to rats in their food is also known to induce CNS (5) and peripheral nerve (6) demyelination. Likewise, subcutaneously injected cyanide produces demyelinated lesions in the CNS (7). In addition to these studies, it seemed a reasonable corollary to extend them to EAE, which is considered to be a reasonable working model for certain aspects of the human disease multiple sclerosis. EAE is classified as a demyelinating disease in rodents and is characterized by a marked flaccid paralysis of the hind limbs, in the rat a decreasing tendency of the tail to curl around the examiner's finger, waddling gait, incontinence and death in the most severely affected animals (8).

MATERIALS AND METHODS

Preparation of rats and determination of urinary ^{14}C. IC injection of [4-^{14}C]cholesterol into 10- to 12-day-old Sprague-Dawley rats and collection of urine for ^{14}C determination have been described (1). The Lewis rats used in the present experiments were similarly treated. ^{14}C was determined on a Packard Tricarb Model 3320 scintillation counter (Packard Instrument Co., Downers Grove, IL) with an efficiency of 85% for ^{14}C. Samples containing 0.5 mL of urine and 10 mL of Instagel (Packard Instrument Co.) were used for measurements. [4-^{14}C]Cholesterol IC injected Lewis rats were used for the EAE studies, and Sprague-Dawley rats were used for all other experiments. All rats were subjected to demyelination experiments only when they reached six months of age or more, and all weighed 250 g or more at the beginning of the experimental period. All animals showed weight loss by the time of sacrifice. The age of the rats used to determine the distribution of ^{14}C in tissues is indicated in the appropriate tables. All rats were maintained on Purina rat chow (Ralston-Purina, St. Louis, MO) and water *ad libitum*.

Chemicals and other materials. TET(Cl) was purchased from KKK Pharmaceuticals (Plainview, NY). HCP was purchased from the Aldrich Chemical Company (Milwaukee, WI). Sodium cyanide, analytical grade, was purchased from Fisher Scientific Company (St. Louis, MO). Soluene-350 was purchased from Packard. Sprague-Dawley rats were purchased from the St. Louis University School of Medicine, and Lewis rats were purchased from Charles River Laboratories (Wilmington, ME). *Myobacterium tuberculosis* H37RA and Freund's Incomplete Adjuvant were obtained from Difco

Laboratories (Detroit, MI). Guinea pig spinal cord was obtained from locally purchased animals.

Administration of chemical demyelinating agents. TET(Cl) [$Sn(C_2H_5)_3Cl$] was presented to rats in their drinking water at 5 mg/L initially, then at 10 mg/L to accelerate the pathological process. HCP was pulverized and mixed with finely ground rat chow at a concentration of 50 mg or 100 mg/100 g of chow. The mixture was pounded into 2 × 10 cm rods and placed in troughs in metabolic cages to minimize contamination of food with urine. Sodium cyanide was subcutaneously injected into the abdomen as either 1.0 mg or 2.0 mg in 0.5 mL water.

Induction of EAE. Lewis rats age 10- to 12-d were injected IC with [4-^{14}C]cholesterol as previously described (1). Six months after labeling, the rats were placed into metabolic cages for urine collection. Prior to induction of EAE, a somewhat longer than usual control period was allowed for collection of urine. The rats were injected intradermally into both hind feet pads with a suspension of 5.0 mg (wet weight) of whole guinea pig spinal cord plus 3.0 mg of *M. tuberculosis* per mL of Incomplete Freund's Adjuvant. Each rat received a total of 0.25 mL of the mixture at six sites on the hind foot pads.

Examination of tissue for ^{14}C. CNS tissues were suspended in distilled water by homogenization in a close-fitting Teflon-glass pestle, and aliquots were taken for ^{14}C determination. Two methods were used to examine other body tissue. In the first method, the tissues were dried *in vacuo* over potassium hydroxide pellets and then ground as thoroughly as possible using a mortar and pestle (or, for larger organs, a Waring Blender). The ground tissues were then extracted overnight in a Soxhlet extractor with chloroform/ethanol (2:1, vol/vol). The volume of the extracts was reduced (nitrogen, water bath), and an aliquot (evaporated to dryness) was taken for ^{14}C determination. In the second method, the organs or tissues were blotted, weighed and macerated using a mortar and pestle. A weighed portion was dissolved in Soluene-350 (0.5N quaternary ammonium hydroxide) either by standing at room temperature or by heating on a steam bath. Aliquots of the solution (0.5 mL) were added to 10 mL Instagel and counted for ^{14}C. Soluene-350 (0.5 mL) in 10 mL Instagel served for background counts.

Light and electron microscopy. The rats, when received for these studies, were extremely lethargic or moribund. They were anesthetized intraperitoneally with a lethal dose of Diabutal (veterinary sodium barbital). Because of the need for biochemical assay of CNS tissues, it was not possible to perfuse the rats with fixative. The calvarium was opened rapidly and tissue samples were taken from the cortex, cerebellum and spinal cord. This was somewhat difficult because of the markedly edematous condition of the CNS. The tissue samples were placed in 3% glutaraldehyde in Millonig's phosphate buffer for electron microscopic studies and in 10% neutral buffered formalin for light microscopic examination. The tissue for electron microscopy was post-fixed in 1% 0.05 M osmic acid in Millonig's phosphate buffer and processed for Epon p12 embedding. Thin sections were double-stained with lead citrate and uranyl acetate. Tissue for light microscopy was embedded into paraffin and stained by the Weil technique to show myelin. The tissues from two normal Sprague-Dawley rats were compared for control.

Urinary ^{14}C excretion controls. It is difficult to distinguish on routine daily assays between urinary ^{14}C and the radioactive potassium (^{40}K) normally excreted in urine (9). Although there is no indication that ^{40}K urinary excretion is elevated during demyelinating conditions, proper controls seemed advised. Therefore, two Sprague-Dawley rats and two Lewis rats each weighing 250+ g, that were not injected with ^{14}C, were treated with the demyelinating agents. These rats were placed into metabolic cages and fed Purina rat chow *ad libitum*, and their urine was collected daily and counted for ^{14}C. Each rat showed the characteristic pathological indications anticipated for the treatment administered. As an additional control, one Sprague-Dawley rat labeled with ^{14}C was placed in a metabolic cage and given one subcutaneous injection of 0.5 mL saline in the abdomen, and urine was collected and counted for ^{14}C in the manner described.

RESULTS

Experiments with TET(Cl). The urinary ^{14}C excretion patterns of four previously injected rats that were given drinking water containing TET(Cl) are shown in Figure 1. (For comparison, the urinary excretion pattern of a control rat will later be shown in Figure 7A.) The clinical signs we observed with progressing disease were as described by Eto *et al.* (3). The rats at sacrifice were in extremely poor condition, severely emaciated and nearly comatose, with partial or complete paralysis of the hind limbs. Urine excretion was reduced, but not completely eliminated, and there was little or no fecal excretion. Light microscopic examination of CNS tissue revealed edema and vacuolation varying from slight to marked. The myelin stained tissue appeared pale in the white matter with swelling of the glial cells. This picture seemed consistent with spongy degeneration of neural tissue. Electron microscopy showed rather severe splitting at the myelin laminae, with concomitant bleeding or vacuolation of the nerve fibers as well as marked edema and swelling of the astrocytes (light and electron microscope pictures were taken but are not shown). In short, the microscopic picture matched that observed after administration of triethyl tin sulfate (2,3). Figure 1 clearly shows a marked increase in urinary ^{14}C excretion following administration of the demyelinating chemical. (The ^{14}C excretion pattern for a control rat will be shown in Fig. 7A.) As can be noted from Figure 1A, the average daily total counts per minute (cpm) for the two peaks for days 37–46 is 1,090, as compared to the average daily total cpm for days 2–36 of 475 cpm. This represents an increase of 56%. Similarly, in Figure 1C the average daily total cpm of the two peaks for days 37–46 is 1,188 as opposed to the average for days 1–36 of 447, an increase of 62%. If one considers the peaks for days 43–46 (6,879 cpm), the increase amounts to 81%.

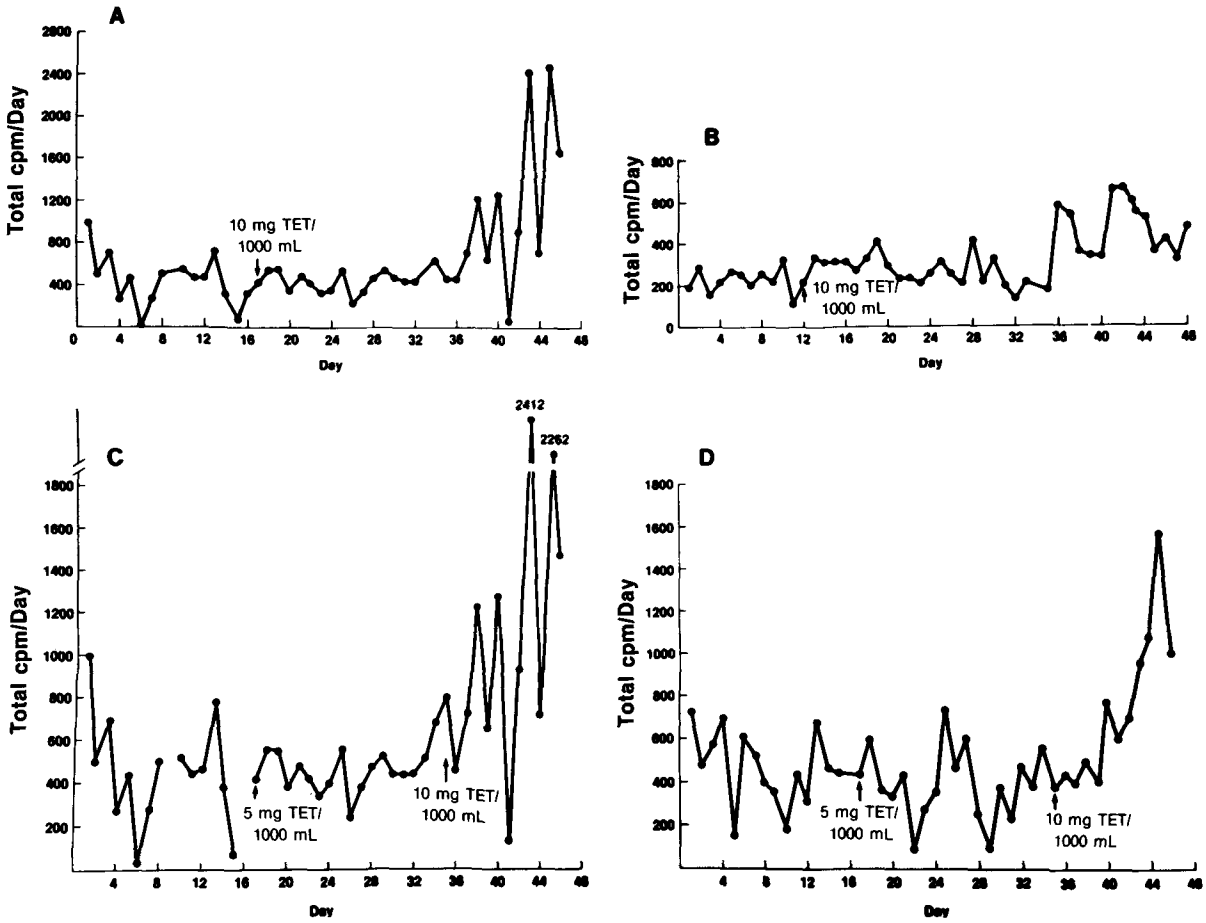

FIG. 1. ^{14}C Urinary excretion pattern of four adult rats each given 5 μCi of [4-^{14}C]cholesterol intracerebrally one year previously, and receiving triethyl tin (TET) chloride in their drinking water as indicated; cpm, counts per minute.

Experiments with HCP. Two rats with CNS prelabeled with [4-^{14}C]cholesterol were given HCP mixed with their chow in the amount of 5 or 10 mg per 100 g of chow. These rats responded as expected (6) with characteristic symptoms (weight loss, general weakness, partial paralysis of the hind limbs). The urinary ^{14}C excretion patterns of the two experimental rats are shown in Figure 2. (For comparison, the urinary excretion pattern of a control rat will be shown later in Fig. 7C.) The excretion pattern for the rat shown in Figure 2B is probably the weak link of all the groups. We suggest, in retrospect, that this is due to the fact that a longer "control period" should have been used, and that less demyelinating agent was taken in by the rat due to the method of administration [eating as opposed to drinking as in the case of the TET(Cl)-treated rats]. Nevertheless, the peak shown for days 22–31 constitutes a 27% increase in ^{14}C urinary excretion over the entire previous period (days 2–21).

Experiments with sodium cyanide. The effects of subcutaneously injected sodium cyanide were investigated using two rats with their CNS prelabeled with [4-^{14}C]-cholesterol. These rats exhibited the characteristic physical responses reported in the literature (7) and showed increased urinary ^{14}C excretion as shown in Figure 3. A massive release of ^{14}C into the urine, beginning several days after injection of NaCN, is apparent. This excretion continued for at least two weeks after administration of NaCN had ceased, subsequently diminished, but did not return to normal levels. Since these rats did not appear to be in pain, the injections were reinstated at 2 mg per day. Several days later there was another marked increase in ^{14}C excretion, and the rats were sacrificed upon showing severe symptoms characteristic of cyanide injection. (The urinary excretion pattern of one control rat is depicted in Fig. 7B.)

Experiments involving induction of EAE. Four Lewis rats with CNS cholesterol labeled with ^{14}C and in which EAE had been induced showed characteristic microscopic lesions in multiple sections of the brain and spinal cord. In addition, all exhibited the extreme hind leg paralysis associated with the disease. The urinary ^{14}C excretion patterns of these rats are shown in Figure 4. In each case a marked increase in ^{14}C excretion was observed following the induction of EAE, analogous to that following the administration of the chemical demyelinating agents previously discussed. The excretion of a control EAE rat will be shown in Figure 7D.)

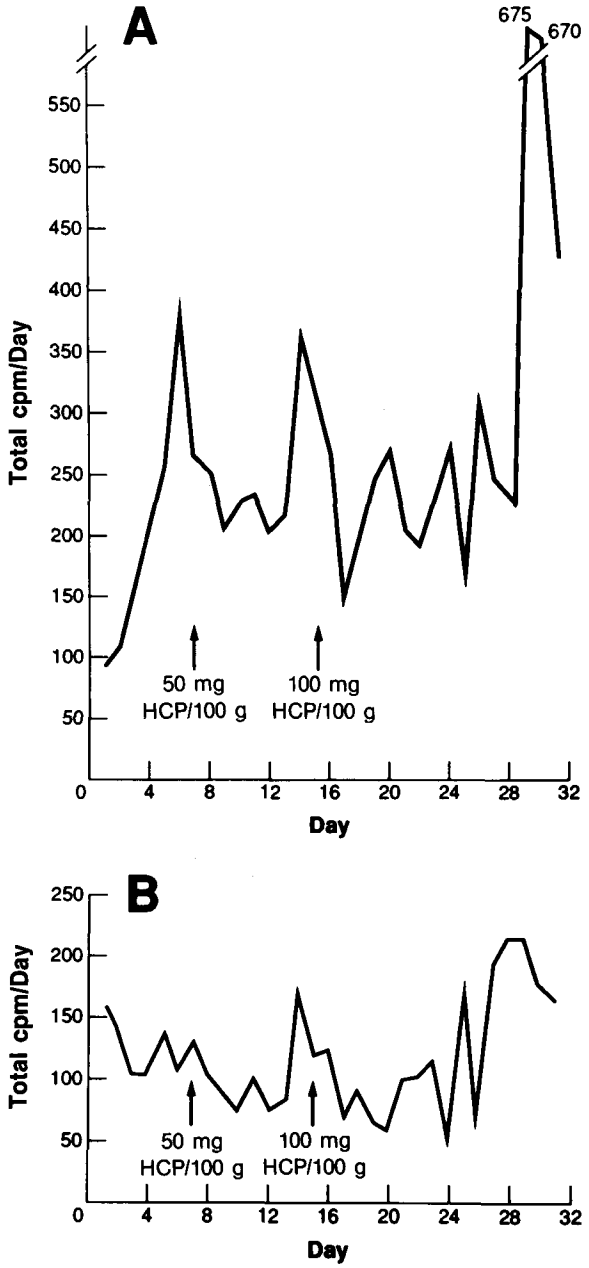

FIG. 2. ^{14}C Urinary excretion pattern of two adult rats given 5 µCi of [4-^{14}C]cholesterol one year previously and hexachlorophene (HCP) in their chow as indicated; cpm, counts per minute.

Distribution of ^{14}C in rats injected IC with [4-^{14}C]cholesterol. The distribution of ^{14}C in CNS subcellular fractions of adult rats injected IC with [4-^{14}C]cholesterol at 10 d of age was reported in an earlier paper (1). Most of the ^{14}C (87%) was associated with myelin. All rats in the current study exhibited approximately the same subcellular distribution of ^{14}C in brain and spinal cord (data not shown). For the present studies and for future work, it seems of interest to present data also showing the distribution of ^{14}C in the total rat.

TABLE 1

Distribution of ^{14}C Label in Central Nervous System (CNS) Sections of an Adult Rat After Intracerebral Injection of [4-^{14}C]Cholesterol (at 12 d of age)

CNS section	Total dpm per region[a]	dpm/mg
Right cerebrum	263,700	1,290
Left cerebrum	292,200	1,328
Cerebellum	152,900	725
Medulla	234,300	1,143
Spinal cord[b]	60,000	517

[a]Measured by suspension of whole region in water with a close-fitting Teflon-glass hand homogenizer from which a 0.5-mL aliquot was taken for ^{14}C determination.
[b]Represents only 0.116 g of upper spinal cord.

Although the initial injections of ^{14}C were into one cerebral hemisphere, the ^{14}C permeated the CNS (see Table 1). Two months after IC injection of [4-^{14}C]cholesterol, there was still a considerable amount of ^{14}C in the major organs (Table 2). However, six months after IC injection, only the intestine and liver (other than the CNS) showed evidence of ^{14}C, and this only in traces (Table 2). Two years after IC injection, no ^{14}C was detected in any organ tissue except the CNS (also Table 2). The trace

TABLE 2

^{14}C Content of Rat Tissues at Time Intervals Following Intracerebral Injection of [4-^{14}C]Cholesterol (at 12 d of age)

			Two years	
Tissue	Two months[a] (dpm/mg)[c]	Six months[b] (dpm/mg)[c]	Extract of whole organ[d]	dpm/mg
Brain	1,571	1,600	34,500	14
Spinal cord	—	—	3,500	6
Skull	—	—	600[e]	—
Intestine (washed)	9	5	0	—
Kidney	13	0	0	—
Stomach	—	—	0	—
Lungs	—	—	—	—
Liver	2	5	—	—
Heart	2	0	—	—
Blood[f]	0	0	0	—
Spleen	—	0	0	—
Carcass (including skin)	—	0	100	—

[a]Data taken from one of three rats injected intracerebrally with 5 µCi of [4-^{14}C]cholesterol at 12 d of age.
[b]Data taken from one of two rats injected intracerebrally with 5 µCi of [4-^{14}C]cholesterol at 12 d of age.
[c]Work-up of tissues for ^{14}C determination given in Materials and Methods section (Soluene-350 method).
[d]Determined after chloroform/methanol (2:1, vol/vol) extraction. See Materials and Methods section for details.
[e]Results believed to be due to residual central nervous system tissue not removed from spinal cord and skull.
[f]Five-mL sample taken at time of sacrifice.

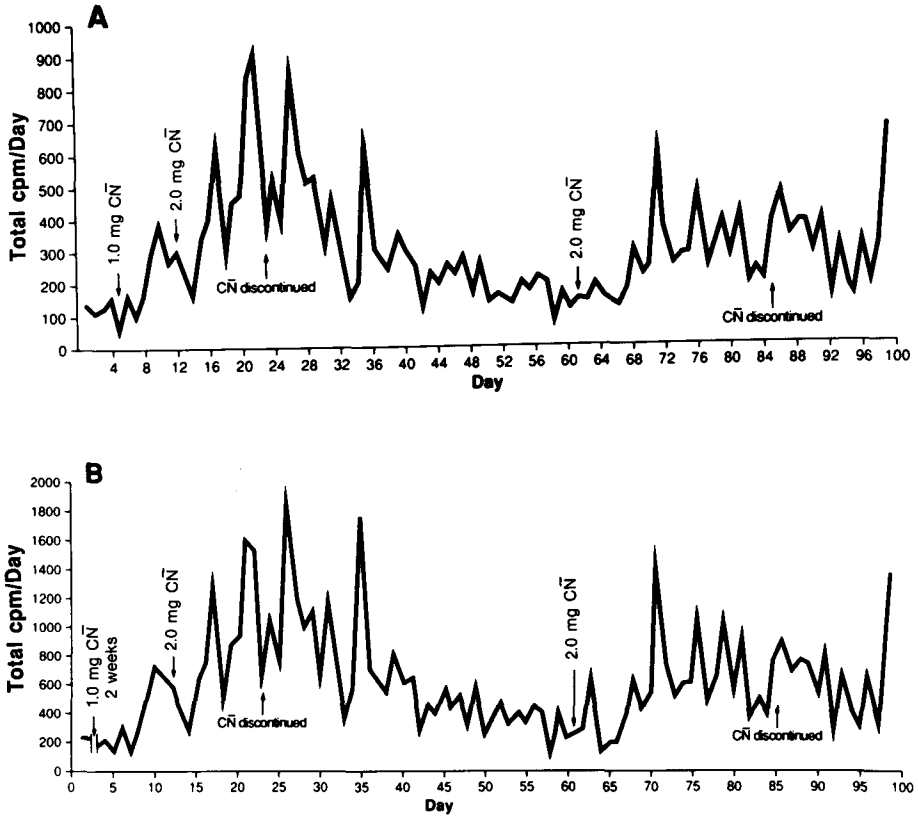

FIG. 3. ^{14}C Urinary excretion pattern of two adult rats given 5 μCi of [4-^{14}C]cholesterol intracerebrally one year previously and sodium cyanide (CN̄) subcutaneously as indicated. Despite the remarkable similarity of the two patterns, the data are from two different rats (e.g., see ordinate values); cpm, counts per minute.

amount detected in the carcass was negligible. The absence of ^{14}C in the kidney six months after injection illustrates the transient role of this organ in the excretory process. Therefore, from the data shown, it is apparent that the urinary ^{14}C measured must have come from the CNS, at least initially.

The effect of starvation and subcutaneous injection on the urinary excretion of ^{14}C. As the work progressed, the question arose as to whether other experimental conditions might influence the excretion of urinary ^{14}C. While we could not cover every contingency, two were considered for this study. For example, we wondered whether the single subcutaneous injection as used in the cyanide experiments might have an effect. Figure 5 suggests that it does not, since there was no change in urinary ^{14}C level after subcutaneous injection of 0.5 mL of saline. This also serves as an additional baseline control. More critical, however, was the potential effect of lowered food intake common to all of the animals in these experiments, resulting in measurable weight loss. The effect of starvation, which is readily reproducible, is shown for one rat in Figure 6. Instead of an increase in urinary ^{14}C excretion, there was an immediate drop. Whether this is in response to the CNS or the kidneys (or both) is presently not known.

Controls and ^{14}C baselines. Controls for each demyelinating experiment, using nonlabeled rats, are shown in Figure 7. There was no increase in urinary ^{14}C under any of the demyelinating conditions tested. It should be noted that the baselines for all of these controls are considerably lower than that seen for the rats with ^{14}C-labeled brain and spinal cord. This is because the nonlabeled rats excreted only ^{40}K. Urinary ^{14}C excretion in labeled rats is characterized by peaks even in the pretreatment periods and is markedly increased in the experimental periods. This suggests some rhythmic type of excretion, the significance of which is unknown at this time.

DISCUSSION

A large number of chemicals are known to be toxic to the CNS by causing demyelination (10). Of these we chose three for the present study because they are easily administered to animals *via* diet or by injection. EAE was chosen because of its analogy to multiple sclerosis.

Although a small amount of ^{14}C is eliminated from the CNS *via* the biliary route (Nicholas, H.P., Elliott, W.H., and Hsu, F., manuscript in preparation), the major route of excretion from brain and spinal cord, as determined by our tracer studies, is the urinary route.

There are several important points that can be made based on our studies. First, a large increase in urinary cholesterol metabolites results when CNS demyelina-

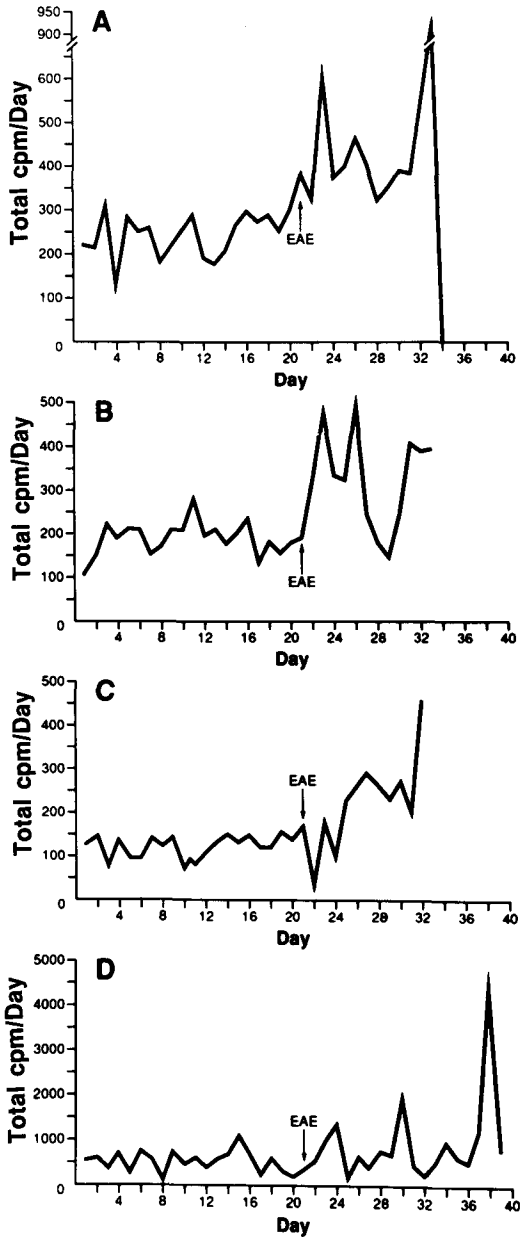

FIG. 4. ^{14}C Urinary excretion pattern of four adult rats given 5 µCi of [4-^{14}C]cholesterol intracerebrally one year previously and experimental allergic encephalomyelitis (EAE) as indicated. The arrows shown indicate the day they were injected intradermally in both hind feet pads with whole guinea pig spinal cord plus *Myobacterium tuberculosis* in Incomplete Freund's Adjuvant; cpm, counts per minute.

FIG. 5. ^{14}C Urinary excretion pattern of an adult rat given 5 µCi of [4-^{14}C]cholesterol intracerebrally one year previously and a single subcutaneous injection of 0.5 mL saline in the abdomen as indicated; cpm, counts per minute.

FIG. 6. ^{14}C Urinary excretion pattern of an adult rat given 5 µCi of [4-^{14}C]cholesterol intracerebrally one year previously, with food removed as indicated; cpm, counts per minute.

tion occurs. Therefore, this reflects the degree of CNS demyelination and can probably be determined quantitatively by chemical means in nonlabeled species (e.g., humans). Secondly, the metabolites, if formed within the CNS, could have considerable physiological (or pathological) significance because they are excreted in greater quantity under demyelinating conditions. One of these constituents is an acidic steroid that could by itself be responsible for demyelination (11–13).

One group of urinary metabolites has been described (1). A second group of the same type, but differing in solubility and polarity, has been identified (manuscript in preparation). The precise nature of the metabolites may differ with the demyelinating agent. Additional studies are necessary to determine this.

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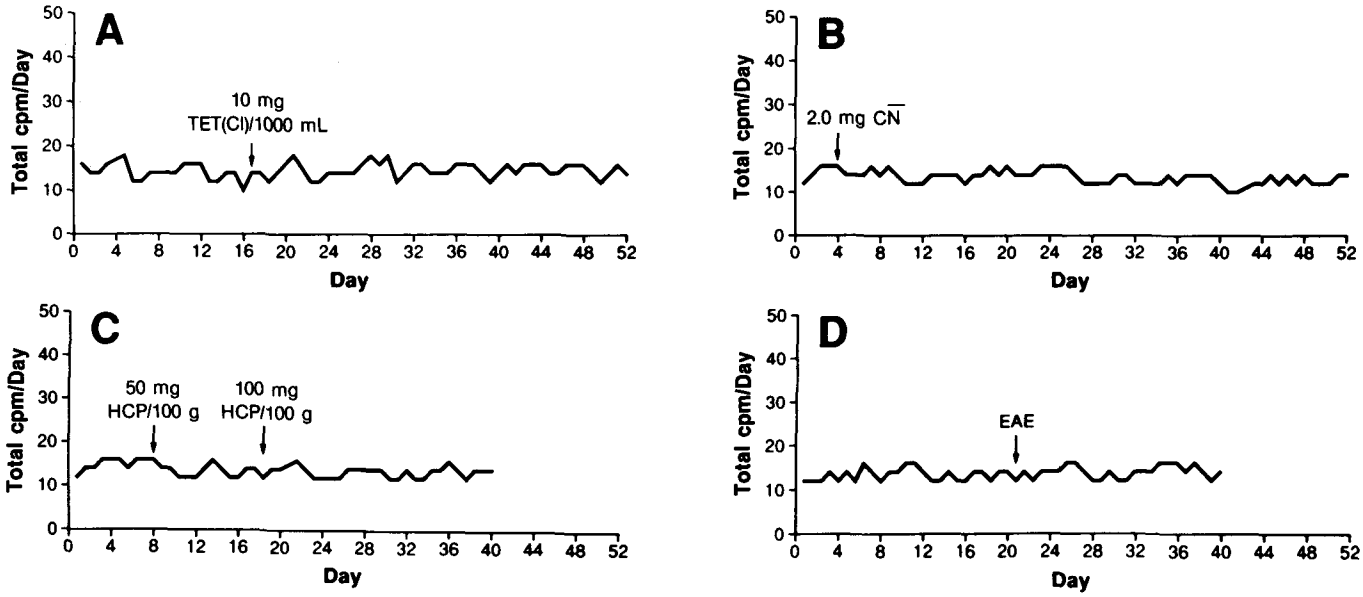

FIG. 7. ^{14}C Urinary excretion pattern of controls for the experiments shown in Figures 1–4. Two rats for each group were used, but the excretion pattern of only one rat is shown because the respective curves were for all practical purposes superimposable. The arrows indicate where the disease processes were initiated. $C\bar{N}$, sodium cyanide; HCP, hexachlorophene; EAE, experimental allergic enaphalomyelitis. See Figure 1 for other abbreviations.

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Dietary Lipids Modify the Fatty Acid Composition of Cartilage, Isolated Chondrocytes and Matrix Vesicles

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The effects of dietary lipids on the fatty acid composition of hyaline cartilage, epiphyseal chondrocytes (EC) and matrix vesicles (MV) were evaluated in chicks. A basal semipurified diet was fed to chicks containing one of the following lipid sources at 70 g/kg: soybean oil, butter + corn oil, margarine + corn oil or menhaden oil + corn oil (MEC). Articular and epiphyseal growth cartilage were isolated from the proximal tibiotarsus; EC and MV were subsequently released by trypsin (EC 3.4.21.4) and collagenase (EC 3.4.24.3) digestion followed by ultracentrifugation. The fatty acid composition of polar lipids in chick epiphyseal cartilage at three and six weeks, as well as articular cartilage, EC and MV at eight weeks of age revealed the presence of high levels of saturated and monounsaturated fatty acids (up to 85.5%) but low levels of n-6 polyunsaturated fatty acids (PUFA) (2.6–10.2%). Mead acid (20:3n-9, >3%) was also present in cartilage, EC and MV lipids, and was unaffected by the dietary lipid treatments. Total n-3 PUFA concentrations were the highest in cartilage, EC and MV of chicks consuming MEC. Feeding MEC lowered the levels of 20:4n-6 in cartilage, but increased 20:5n-3 levels. The data are consistent with those reported previously which showed that cartilage tissues are low in n-6 PUFA and that they contain 20:3n-9. We furthermore demonstrated that the PUFA composition of cartilage can be modified by dietary lipids.
***Lipids 29,* 619–625 (1994).**

Matrix vesicles (MV) are believed to be responsible for initiating *de novo* mineralization in most calcifying tissues (1). The membrane-bound acidic phospholipids located within the MV structure facilitate mineralization by forming lipid–Ca^{2+}–protein complexes (2). High alkaline phosphatase (AP) activity is present in isolated MV (3), and this activity is much higher than that found in chondrocytes.

Peress *et al.* (4) first studied the lipid composition of the MV fraction isolated from epiphyseal cartilage of fetal calves. Wuthier (5) then reported the fatty acid composition of lipids of epiphyseal chondrocytes (EC) and MV obtained from chicken growth plate cartilage. Polyunsaturated fatty acids (PUFA) are structurally and physiologically important for mineralizing tissues in bone and for locally regulating bone modeling. Fatty acids serve as precursors for the synthesis of the phospholipids present in MV, and PUFA also serve as substrates for prostaglandin (PG) E_2 biosynthesis. PGE_2 produced in bone tissue acts as a potent bone resorbing agent and appears to regulate the activity of cytokines and growth factors in bone (6). Recently, Adkisson *et al.* (7) discovered high concentrations of 20:3n-9 and low concentrations of n-6 PUFA in normal cartilage tissues of several vertebrates, including in humans and in chicken. However, the impact of dietary lipids on bone, cartilage and MV function and metabolism is presently unknown, even though it is well established that dietary lipids can modulate n-6 and n-3 PUFA levels in *Aves* (8).

In the present study, various dietary lipids varying in PUFA composition were evaluated in chicks to determine their effects on the fatty acid composition of cartilage, EC and MV. A basal semipurified diet which contained varying amounts of n-6 or n-3 PUFA, saturated fatty acids or *trans*-18:1 fatty acids was fed to chicks.

MATERIALS AND METHODS

Animals and diets. One-day-old Hubbard Cockerel broiler chicks obtained from a commercial hatchery were fed a semipurified diet (Table 1) formulated to meet all of the nutrient requirements for chick growth (9). One of four dietary lipid treatments were given at 70 g/kg of diet: soybean oil (SBO) (Bunge Foods Inc., Bradley, IL), butter (75%) + corn oil (25%) (BC), margarine (80%) + corn oil (20%) (MAC) or menhaden oil (57%) + corn oil (43%) (MEC). Menhaden oil was donated by Zapata Haynie Co. (Reedville, VA); butter (Weaver Brothers Inc., Roanoke, IN), margarine (Scot Lad Foods Inc., Pewaukee, WI) and corn oil (Best Foods, CPC International Inc., Englewood Cliffs, NJ) were purchased locally. Corn oil was added to all lipid treatments (except SBO) to supplement linoleic acid. Each diet was given to four replicate pens of ten chicks. Diets and water were provided *ad libitum.*

Collection of cartilage and isolation of chondrocytes and MVs. Three-, six- and eight-week-old chicks were killed by exsanguination. Tibiotarsal bones with intact articular and epiphyseal cartilage and liver were collected, chilled on ice, and stored at –20°C. EC and MV from epiphyseal cartilage were isolated immediately after collecting tibiotarsal bones from eight-week-old chickens following the method described by Genge *et al.* (10). Briefly, the growth cartilage tissues were finely sliced (0.1–0.3-mm thick) and digested in ice-cold, synthetic cartilage lymph (SCL), as described by Majeska and Wuthier (11), containing 0.1% trypsin (EC 3.4.21.4; 4 mL/g tissue; Sigma Chemical Co., St. Louis, MO) at 37°C for 15 min. After removing the trypsin buffer, cartilage tissues were digested in SCL buffer containing crude collagenase (EC 3.4.24.3; 500 units/g tissue; Sigma) at 37°C for 5 h with shaking. The

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Abbreviations: AP, alkaline phosphatase; BC, butter + corn oil diet; EC, epiphyseal chondrocytes; FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; MAC, margarine + corn oil diet; MEC, menhaden oil + corn oil diet; MV, matrix vesicles; PG, prostaglandin; PUFA, polyunsaturated fatty acids; SBO, soybean oil diet; SCL, synthetic cartilage lymph; TES, *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Composition (wt%) and Ingredient Composition of the Experimental Diets[a,b,c]

Fatty acid	Dietary sources			
	SBO	BC	MAC	MEC
14:0	0.08	7.29	0.09	3.74
16:0	9.72	24.50	9.78	13.20
16:1n-7	0.09	0.34	0.08	5.56
18:0	4.34	11.51	8.50	2.68
*t*18:1	ND[d]	2.19	14.85	ND
18:1	24.92	26.57	24.22	17.08
18:2n-6	50.00	18.38	34.17	26.66
18:3n-3	6.55	0.90	3.03	1.05
20:0	0.37	0.29	0.41	ND
20:1n-9	0.24	0.11	0.18	0.72
20:5n-3	ND	ND	ND	9.36
22:5n-3	ND	ND	ND	1.99
22:6n-3	ND	ND	ND	5.83
SAT	15.06	45.03	19.27	21.06
MONO	25.27	31.29	39.37	25.19
PUFA	56.55	19.99	38.03	47.58
n-6 PUFA	50.00	19.09	35.00	27.61
n-3 PUFA	6.55	0.90	3.03	19.97
n-3/n-6 ratio	0.13	0.05	0.09	0.72

[a]The semipurified basal diet contained (g/kg): isolated soybean protein, 250; corn starch, 283.75; dextrose, 283.75; lipid, 70; cellulose, 30; $CaHPO_4 \cdot 2H_2O$, 25; $CaCO_3$, 15; DL-methionine, 7.5; mineral premix, 30; vitamin premix, 5. The diet was formulated to contain 22.5% crude protein and 3557 kcal/kg metabolizable energy. The analyzed crude protein was 23.5%, and lipid content was 6.5–7.8% for the four dietary treatments. SBO, soybean oil diet; BC, butter + corn oil diet; MAC, margarine + corn oil diet; MEC, menhaden oil + corn oil diet; SAT, saturated fatty acids; MONO, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[b]Mineral premix provided (mg/kg diet): ZnO, 90; $MgSO_4 \cdot 7H_2O$, 6000; $MnSO_4 \cdot H_2O$, 300; $CuSO_4 \cdot 5H_2O$, 60; $FeSO_4 \cdot 7H_2O$, 500; KIO_3, 6; $Na_2MoO_4 \cdot 2H_2O$, 10; $CoCl_2 \cdot 6H_2O$, 5; Na_2O_3Se, 0.43; KCl, 1500; K_2HPO_4, 6000; NaCl, 6000.

[c]Vitamin premix provided per kg of diet: vitamin A palmitate, 4500 IU; cholecalciferol, 4500 ICU; menadione sodium bisulfite, 1.5 mg; DL-α-tocopherol, 50 IU; thiamine hydrochloride, 15 mg; riboflavin, 15 mg; sodium pantothenate, 50 mg; niacin, 50 mg; pyridoxine hydrochloride, 6 mg; folic acid, 6 mg; cyanocobalamin, 0.02 mg; *d*-biotin, 0.3 mg; choline chloride, 600 mg; butylated hydroxytoluene, 200 mg.

[d]ND, not detected.

digest was gently vortexed for 3 min to release unbound chondrocytes and then filtered through nylon mesh to remove debris. The filtrate was centrifuged at 250 × *g* for 20 min, and the pellet containing the chondrocyte fraction was resuspended with *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer (50 mM TES, 1.4 mM $MgCl_2$, 10% sucrose, pH 7.5). The supernatant was centrifuged at 13,000 × *g* for 30 min to remove the membrane fragments and mitochondria, and then at 100,000 × *g* for 60 min to sediment the MV. The MV pellet was resuspended in TES buffer.

Total protein measurement. Total protein was determined using a protein assay kit (purchased from Sigma, P 5656) which is based on the method of Lowry *et al.* (12).

AP assay. AP activity in EC and MV was measured as described by Tietz *et al.* (13). *p*-Nitrophenyl phosphate (USB Co., Cleveland, OH) was used as substrate to assay AP activity. AP specific activity was defined as μmol of *p*-nitrophenyl phosphate hydrolyzed/mg protein/min.

Fatty acid analysis. Lipids in the diet, in liver, in tibiotarsal articular and epiphyseal cartilages, as well as in EC and MV were extracted with chloroform/methanol (2:1, vol/vol). The polar lipids from liver and cartilage were isolated by solid-phase extraction (14). Lipids (except from articular cartilage) were saponified, and fatty acid methyl esters (FAME) prepared using 14% boron trifluoride in methanol (Alltech Associates Inc., Deerfield, IL). FAME were analyzed using a gas chromatograph (HP 5890 series II, autosampler 7673, HP 3365 ChemStation; Hewlett-Packard Co., Avondale, PA) which was equipped with a DB 23 column (length 30 m, i.d. 0.53 mm, film thickness 0.5 μm; J&W Scientific Co., Folsom, CA) and was operated at 170°C for 10 min, with the temperature programmed 1°C/min to 210°C and held for 5 min. The injector and flame-ionization detector temperatures were 225 and 250°C, respectively. FAME were identified by comparison of their retention times with those of standards (GLC 422, Nu-Chek-Prep, Elysian, MN) and of FAME prepared from menhaden oil (18:4n-3 and 20:5n-3). Fatty acid compositions are given as weight or area percentages. *Cis* and *trans* 18:1 FAME were quantified after separation on silver-nitrate impregnated thin-layer chromatography (TLC) plates followed by capillary gas–liquid chromatography (GLC) (15). Mead acid was isolated in the triene band by argentation TLC (16), recovered, and quantified by capillary GLC. The polar lipids of articular cartilage were saponified (10% KOH in methanol), and fatty acid pentafluorobenzyl esters were prepared (17) and quantified by capillary GLC (SP-2380, length 30 m, i.d. 0.32 mm, film thickness 0.20 μm; Supelco, Inc., Bellefonte, PA). The gas chromatograph (HP Model 5890) was held at 160°C for 2 min, temperature programmed at 10°C/min to 200°C, and then held for 10 min. Both injector and electrochemical detector temperatures were set at 225°C.

Statistical analysis. Data were subjected to analysis of variance, and where significant differences were found a Duncan's multiple range test at a probability of $P < 0.05$ was performed (18). Variation within treatments is expressed as the pooled standard deviation (SD) or pooled standard error of the mean (SEM).

RESULTS

Chick growth and tibiotarsal bone length were not affected by the different dietary lipid treatments. The MV were characterized by high AP activity (12 μmol/mg protein/min) as compared to the activity found in EC (0.4 μmol/mg protein/min) and confirmed by transmission electron microscopy. The AP activities in the EC and MV were not affected by the different dietary lipid treatments.

The fatty acid compositions of the tibiotarsal epiphyseal cartilage in three- and six-week-old chicks, and of EC, MV and articular cartilage in eight-week-old chicks showed that in polar lipids the total saturated and monounsaturated fatty acids were high (>80%) and the total n-6 PUFA were very low (<10.2%). The 18:1 peak seen in GLC represented primarily *c*18:1n-9 and *c*18:1n-7.

The fatty acid compositions of polar and neutral lipids of epiphyseal cartilage of chicks were affected by the lipids fed (Tables 2 and 3). Chicks consuming the MEC diet had the highest amounts of total n-3 PUFA and the highest n-3/n-6 PUFA ratios in polar and neutral lipids at three and six weeks of age. The amounts of 20:5n-3, 22:5n-3 and 22:6n-3 in polar and neutral lipids of epiphyseal cartilage seemed to increase from three to six weeks in chicks fed MEC as compared with chicks fed the other diets. At six weeks, chicks given MEC had the lowest levels of 20:4n-6 and 22:4n-6 in their polar lipids (Table 3). The amounts of 14:0, 16:0 and 18:1 were higher in polar lipids of epiphyseal cartilage of chicks consuming BC compared to the values obtained from chicks given SBO. The amounts of 18:0 were highest in the neutral lipids of cartilage of chicks fed BC at three weeks, and the total amounts of stearic acid in cartilage decreased from three to six weeks independent of the dietary treatment. At six weeks, the total amounts of PUFA (n-6 and n-3) were lowest in cartilage neutral lipids of chicks fed BC, reflecting the fatty acid composition of the BC diet (Table 3).

When cartilage lipids were analyzed by GLC, a major peak was observed having a retention time identical to that of 20:2n-6. Further characterization by TLC followed by GLC of the diene and triene bands revealed that 20:3n-9 greatly exceeded the amount of 20:2n-6 present in this location. As the components co-eluted, we report here the combined value. The amount of 20:3n-9 in cartilage polar lipids decreased between three and six weeks in all chicks. Neutral lipids of cartilage contained 20:2n-6 and 20:3n-9, but the amounts were higher in chicks at six weeks (Table 3).

The amounts of saturated fatty acids and n-6 PUFA (mainly linoleate) appeared to increase in cartilage lipids

TABLE 2

Fatty Acid Compositions (area %) of Polar Lipids and Neutral Lipids Isolated from Proximal Tibiotarsal Growth Cartilage of Three-week-old Chicks

	Polar lipids					Neutral lipids				
	Dietary lipid treatments				Pooled	Dietary lipid treatments				Pooled
Fatty acid	SBO	BC	MAC	MEC	SEM	SBO	BC	MAC	MEC	SEM
14:0	0.28^{b}	0.51^{a}	0.25^{b}	$0.36^{a,b}$	0.04	$1.99^{a,b}$	2.66^{a}	1.26^{b}	$1.64^{a,b}$	0.22
14:1n-5	0.24	0.15	0.12	0.14	0.04	ND	ND	ND	ND	—
15:0	0.50	ND[c]	ND	0.22	0.12	ND	ND	0.10	0.15	0.06
15:1	4.32	4.04	4.13	4.24	0.34	0.15	0.14	0.48	1.26	0.45
16:0	7.16^{b}	13.63^{a}	$9.52^{a,b}$	$11.22^{a,b}$	1.05	38.31^{a}	25.28^{b}	22.32^{b}	21.62^{b}	2.54
*t*16:1n-7	0.94^{b}	2.04^{a}	1.09^{b}	1.46^{b}	0.16	1.30	1.50	1.33	1.67	0.17
16:1n-7	6.28^{b}	9.18^{a}	6.29^{b}	$7.61^{a,b}$	0.56	5.97	6.30	5.56	7.44	0.54
17:0	0.22^{a}	ND	0.23^{a}	0.14^{b}	0.04	0.24	0.44	0.45	0.35	0.23
17:1	3.27	3.20	3.63	3.41	0.30	ND	ND	ND	ND	—
18:0	10.78^{a}	7.07^{b}	11.86^{a}	8.09^{b}	0.33	9.69^{b}	24.90^{a}	10.28^{b}	12.81^{b}	2.89
18:1	34.25^{b}	38.85^{a}	$35.38^{a,b}$	38.78^{a}	0.94	24.86	32.40	31.51	34.25	2.54
*t*18:2n-6	1.29	1.43	1.13	1.37	0.04	ND	ND	0.64	ND	0.22
18:2n-6	1.20^{b}	1.18^{b}	2.96^{a}	1.00^{b}	0.13	5.44	4.77	6.98	6.40	0.90
18:3n-3	0.22	0.53	0.19	0.21	0.17	0.30	ND	0.34	ND	0.08
18:4	1.22	ND	0.08	ND	0.27	ND	ND	ND	ND	0.12
18:4n-3	ND	ND	ND	0.04	0.02	0.58	ND	ND	0.09	0.15
20:0	1.24	0.06	0.30	0.21	0.28	0.30	0.32	0.45	0.74	0.25
20:1	1.16	0.65	0.72	0.51	0.15	ND	ND	0.29	0.37	0.14
20:2 + 20:3[d]	9.14	9.12	8.28	9.14	0.66	0.52	ND	ND	1.40	0.33
20:4n-6	1.13^{b}	1.02^{b}	2.84^{a}	0.26^{b}	0.25	ND	ND	1.30	ND	0.16
20:5n-3	0.56^{a}	ND	0.10^{b}	0.48^{a}	0.16	ND	ND	0.12	1.17	0.32
22:0	ND	ND	ND	ND	—	ND	ND	ND	0.67	0.18
22:1n-9	0.34	ND	0.10	0.05	0.09	ND	ND	ND	ND	—
22:4n-6	ND	ND	0.47	ND	0.10	ND	ND	ND	ND	—
22:5n-3	ND	ND	0.22^{b}	0.81^{a}	0.13	ND	ND	ND	ND	—
22:6n-3	ND	ND	0.56^{b}	0.96^{a}	0.06	ND	ND	ND	0.50	0.18
24:1n-9	ND	ND	0.19	0.11	0.09	ND	ND	ND	ND	—
SAT	20.15	21.27	22.16	20.24	0.97	50.52	53.60	34.84	37.97	4.54
MONO	50.46^{b}	58.11^{a}	51.65^{b}	54.74^{a}	1.14	32.27	40.34	39.16	44.97	3.11
PUFA	14.76	13.28	16.65	14.32	1.37	6.83	4.77	9.38	9.56	1.44
n-3 PUFA	$0.78^{a,b}$	0.53^{b}	$0.89^{a,b}$	2.55^{a}	0.53	0.87^{b}	0	0.46^{b}	1.76^{a}	0.34
n-6 PUFA	3.62^{b}	3.63^{b}	7.40^{a}	2.63^{b}	0.04	$5.96^{a,b}$	4.77^{b}	8.92^{a}	$7.79^{a,b}$	1.08
n-3/n-6 Ratio	0.21^{b}	0.15^{b}	0.11^{b}	0.96^{a}	0.25	0.11^{b}	0	0.05^{b}	0.21^{a}	0.04

[a,b]Mean values (n = 4) within rows having different superscripts are significantly different ($P < 0.05$).
[c]ND, not detected; for other abbreviations, see Table 1.
[d]Includes 20:2n-6 and 20:3n-9 (Mead acid).

TABLE 3

Fatty Acid Compositions (area %) of Polar Lipids and Neutral Lipids Isolated from Proximal Tibiotarsal Growth Cartilage of Six-week-old Chicks

	Polar lipids					Neutral lipids				
	Dietary lipid treatments				Pooled	Dietary lipid treatments				Pooled
Fatty acid	SBO	BC	MAC	MEC	SEM	SBO	BC	MAC	MEC	SEM
14:0	0.70^{b}	0.88^{a}	$0.77^{a,b}$	0.83^{a}	0.03	0.61	1.15	0.69	0.24	0.26
14:1n-5	0.26	0.30	0.24	0.27	0.02	1.19^{b}	2.61^{a}	0.86^{b}	2.14^{a}	0.16
15:0	0.05^{c}	0.09^{a}	$0.06^{b,c}$	$0.08^{a,b}$	0.01	$0.08^{b,c}$	0.27^{a}	0.02^{c}	0.14^{b}	0.02
15:1	$2.32^{a,b}$	$2.24^{a,b}$	2.11^{b}	2.66^{a}	0.12	0.43	0.11	0.33	0.22	0.17
16:0	20.84	21.93	22.24	22.07	0.46	30.75	36.42	30.72	23.15	3.96
16:1	10.90	11.20	10.29	10.33	0.53	7.06	7.44	7.12	9.32	0.91
17:0	0.05	0.09	0.06	0.11	0.02	0.21	0.74	0.24	1.89	0.78
17:1	1.94	1.64	1.86	2.11	0.11	ND	ND	ND	ND	—
18:0	9.31	8.18	8.71	9.26	0.31	5.27	5.35	5.56	5.90	0.60
18:1	35.01^{a}	35.58^{a}	34.83^{a}	32.13^{b}	0.64	29.08	27.75	30.03	30.93	2.59
*t*18:2n-6	ND[c]	1.01	0.95	ND	0.05	0.50^{a}	0.10^{b}	0.61^{a}	0.59^{a}	0.09
18:2n-6	$4.62^{a,b}$	3.55^{b}	5.08^{a}	3.44^{b}	0.35	13.07^{a}	8.41^{b}	$10.27^{a,b}$	12.60^{a}	0.78
18:3n-3	0.06	0.01	0.04	ND	0.02	1.18^{a}	0.37^{b}	0.58^{b}	0.48^{b}	0.06
18:4	0.36	0.31	0.24	0.40	0.01	ND	ND	ND	ND	—
18:4n-3	ND	0.02	0.02	0.03	0.01	ND	ND	ND	ND	—
20:0	0.20	0.20	0.09	0.19	0.03	0.10	0.05	ND	ND	0.04
20:1n-9	0.63	0.68	0.62	0.55	0.03	0.47	0.30	0.48	0.49	0.07
20:2 + 20:3[e]	3.31	3.47	3.20	3.36	0.23	1.25^{a}	0.34^{b}	1.68^{a}	1.59^{a}	0.21
20:3n-6	0.63^{a}	$0.51^{a,b}$	$0.49^{a,b}$	0.30^{b}	0.06	0.55	0.13	0.28	0.09	0.12
20:4n-6	2.56^{a}	2.21^{a}	2.48^{a}	1.00^{b}	0.26	$1.63^{a,b}$	0.79^{b}	2.16^{a}	0.82^{b}	0.24
20:5n-3	ND	ND	ND	1.80	0.05	ND	ND	ND	3.02	0.06
22:0	ND	0.12	ND	0.08	0.02	ND	ND	ND	ND	—
22:1n-9	ND	0.10	ND	ND	0.03	ND	ND	ND	ND	—
22:4n-6	0.70^{a}	0.52^{a}	0.72^{a}	0.20^{b}	0.08	0.26	0.07	0.08	ND	0.05
22:5n-3	0.32^{b}	0.19^{c}	$0.27^{b,c}$	1.46^{a}	0.03	ND	ND	ND	1.57	0.02
22:6n-3	0.38^{b}	0.25^{b}	0.39^{b}	2.24^{a}	0.08	0.06^{b}	ND	ND	1.97^{a}	0.06
SAT	31.15	31.48	31.93	32.61	0.41	37.01	43.99	37.22	31.32	3.71
MONO	51.06	51.73	49.95	48.05	0.88	38.23	38.20	38.82	43.10	3.50
PUFA	12.94	12.05	13.89	14.24	0.65	$18.50^{a,b}$	10.20^{c}	15.66^{b}	22.65^{a}	1.17
n-3 PUFA	0.76^{b}	0.47^{b}	0.73^{b}	5.53^{a}	0.16	1.24^{b}	0.37^{c}	$0.58^{b,c}$	7.05^{a}	0.16
n-6 PUFA	8.51^{a}	7.80^{a}	9.72^{a}	4.94^{b}	0.66	16.01^{a}	9.50^{b}	13.40^{a}	14.01^{a}	2.22
n-3/n-6 Ratio	0.09^{b}	0.06^{b}	0.07^{b}	1.12^{a}	0.02	0.08^{b}	0.04^{b}	0.04^{b}	0.50^{a}	0.01

[a,b,c]Mean values (n = 4) within rows having different superscripts are significantly different ($P < 0.05$).
[d]ND, not detected; for other abbreviations, see Table 1.
[e]Includes 20:2n-6 and 20:3n-9 (Mead acid).

in chicks between three and six weeks. The amount of 18:2n-6 in cartilage neutral lipids of all chicks increased nearly twofold independent of the dietary lipid treatment. Chicks fed BC, which was lowest in 18:2n-6, had the lowest n-6 PUFA levels in neutral lipids (Table 3).

The concentrations of 20:5n-3, 22:5n-3 and total n-3 PUFA, and the n-3/n-6 PUFA ratios were greatest in EC and MV of chicks fed MEC as compared to the values seen for chicks fed the other dietary lipids (Table 4). Chicks fed MEC had a lower concentration of 20:4n-6 and total n-6 PUFA in EC. The MV from chicks fed MEC maintained the lowest amounts of 18:2n-6, and the amounts of 20:4n-6 in MV were lower than for chicks given SBO. Consistent with what was seen for the polar lipids in epiphyseal cartilage of six-week-old chicks (Table 3), the predominant saturated fatty acid in EC and MV was 16:0, followed by 18:0. Appreciable amounts (3%) of 20:3n-9 were also found in cartilage, EC and MV. The Mead acid concentrations in epiphyseal cartilage, EC and MV were unaffected by the dietary lipid treatments.

The articular cartilage of eight-week-old chicks was dramatically affected by the levels of n-3 and n-6 PUFA present in the MEC and SBO diets, respectively (Table 5). The MEC treatment lowered the amounts of 20:4n-6, total PUFA and n-6 PUFA, but elevated the amount of n-3 PUFA (20:5n-3 and 22:6n-3) and the n-3/n-6 PUFA ratio in chick articular cartilage polar lipids. Additionally, the SBO and MEC treatments lowered 20:3n-9 to less than 3% of the total fatty acids. The concentration of 18:2n-6 was greatest in the polar lipids of articular cartilage in chicks fed SBO, reflecting the composition of soybean oil. Interestingly, the 18:2n-6 increase did not result in a parallel increase in 20:4n-6.

Although a considerable amount of *trans*-18:1 fatty acids was present in the MAC diet (14.85% of *t*18:1) (Table 1), and *trans*-18:1 was found in the growth carti-

TABLE 4

Fatty Acid Compositions (area %) of Chondrocytes and Matrix Vesicles Isolated from Tibiotarsal Growth Cartilage of Eight-week-old Chicks

	Chondrocytes					Matrix vesicles				
	Dietary lipid treatments				Pooled	Dietary lipid treatments				Pooled
Fatty acid	SBO	BC	MAC	MEC	SEM	SBO	BC	MAC	MEC	SEM
14:0	1.40	2.61	2.57	2.70	0.93	$1.10^{a,b}$	1.42^{a}	1.34^{a}	0.99^{b}	0.10
14:1n-5	0.16	0.40	0.32	0.16	0.18	0.09^{b}	0.17^{a}	0.12^{b}	0.11^{b}	0.01
15:0	0.48^{a}	0.11^{b}	0.14^{b}	0.15^{b}	0.16	0.08	0.12	0.09	0.11	0.01
15:1	0.33^{b}	0.68^{a}	0.66^{a}	0.74^{a}	0.22	1.24	1.14	1.16	1.15	0.12
16:0	25.89	28.62	31.21	29.58	5.54	$28.56^{a,b}$	33.33^{a}	$29.92^{a,b}$	26.22^{b}	1.95
*t*16:1n-7	1.16	1.09	1.08	0.97	0.33	1.31	1.17	1.24	1.33	0.15
16:1n-7	5.74	5.85	5.78	5.41	1.42	4.84^{b}	$5.47^{a,b}$	$5.20^{a,b}$	6.04^{a}	0.30
17:0	1.06	0.24	0.28	0.89	0.90	1.03	0.31	0.56	0.21	0.39
17:1	3.35	2.36	3.79	2.72	2.66	1.32	1.24	1.31	1.33	0.15
18:0	5.59	5.37	5.30	5.49	1.62	9.32	8.07	8.01	9.34	0.60
18:1	29.39	27.00	25.89	25.48	4.43	31.42	29.13	28.74	31.63	1.57
*t*18:2n-6	0.61	0.56	0.56	0.47	0.36	0.40^{b}	0.76^{a}	0.82^{a}	0.36^{b}	0.10
18:2n-6	4.64	2.54	1.95	1.94	2.55	$0.84^{a,b}$	$1.43^{a,b}$	1.77^{a}	0.51^{c}	0.25
18:3n-6	0.23	0.47	0.48	0.32	0.19	0.15	0.21	0.10	ND	0.04
18:3n-3	0.35	0.05	ND	ND	0.28	0.11	0.01	0.06	ND	0.03
18:4	2.70	3.29	2.52	ND	2.89	ND	ND	ND	ND	—
18:4n-3	ND[d]	ND	ND	2.55	1.28	0.13	0.10	0.07	0.63	0.17
20:0	0.07	0.32	0.09	0.54	0.41	0.41	0.38	0.36	0.45	0.03
20:1n-9	0.39	0.43	0.25	0.39	0.19	0.79	0.45	0.51	0.50	0.15
20:3n-9	2.92	2.04	2.38	2.45	0.78	3.06	2.79	2.91	3.03	0.49
20:3n-6	0.09	0.11	ND	ND	0.11	0.07	0.06	0.06	ND	0.03
20:4n-6	1.10^{a}	0.68^{a}	0.80^{a}	0.10^{b}	0.38	0.81^{a}	$0.67^{a,b}$	$0.68^{a,b}$	0.34^{b}	0.12
20:5n-3	ND	ND	ND	1.12	0.42	ND	ND	ND	0.36	0.02
22:0	0.68	0.82	0.55	0.41	0.38	1.04	1.03	0.99	1.15	0.13
22:1n-9	$0.40^{a,b}$	0.13^{b}	0.62^{a}	$0.23^{a,b}$	0.32	0.20	0.27	0.30	0.29	0.06
22:2n-6	0.53	0.35	0.17	0.41	0.27	0.58	0.67	0.69	0.72	0.10
22:4n-6	0.26	0.17	0.11	ND	0.18	0.29	0.13	0.23	ND	0.05
22:5n-3	0.42^{b}	ND	ND	0.95^{a}	0.19	0.57^{b}	0.06^{c}	ND	1.01^{a}	0.10
22:6n-3	0.09	0.21	0.12	0.61	0.35	0.63	0.57	0.60	0.63	0.14
24:1n-9	0.29	0.19	ND	0.10	0.22	1.55^{a}	$0.97^{a,b}$	0.73^{b}	$1.38^{a,b}$	0.23
SAT	35.18	38.11	40.14	39.77	5.60	41.57	44.66	41.27	38.49	1.98
MONO	41.21	38.12	38.39	36.20	4.77	42.76	40.01	39.31	43.77	2.13
PUFA	13.92	10.46	9.08	10.92	4.53	7.64	7.45	7.99	7.60	0.93
n-3 PUFA	0.86^{b}	0.26^{b}	0.12^{b}	5.23^{a}	1.30	1.43^{b}	0.64^{b}	0.66^{b}	2.63^{a}	0.22
n-6 PUFA	7.45^{a}	$4.87^{a,b}$	$4.06^{a,b}$	3.24^{b}	2.65	$3.15^{a,b}$	3.92^{a}	4.35^{a}	1.94^{b}	0.46
n-3/n-6 Ratio	0.11^{b}	0.05^{b}	0.02^{b}	1.79^{a}	0.46	0.42^{b}	0.17^{b}	0.17^{b}	1.53^{a}	0.15

[a,b,c]Mean values (n = 6) within rows having different superscripts are significantly different ($P < 0.05$).
[d]ND, not detected; for other abbreviations see Table 1.

lage of three-week-old chicks (1.5% of total FAME) fed MAC, no *trans*-18:1 was detected in the EC and MV of these chicks. Also, no *trans*-18:1 was found in growth cartilage of three-week-old chicks that consumed the BC diet (2.19% dietary *t*18:1).

Figure 1 illustrates the changes in the concentrations of n-3 and n-6 PUFA in liver polar lipids of chicks at three and six weeks of age. The values for fatty acids are presented as standardized differences (STD) based on statistical analysis (number of standard error of the mean units). The STD for n-3 and n-6 PUFA show that chicks fed MEC had lower amounts of 18:2n-6 and 20:4n-6 but higher amounts of 20:5n-3 and 22:6n-3 in liver polar lipids compared to chicks given the other treatments. Changes in n-3 and n-6 PUFA concentrations in liver polar lipids were similar to those found in chick cartilage polar lipids.

DISCUSSION

AP is one of many membrane-bound enzymes found in isolated MV, and its high activity is crucial for proper MV function in mineralization (19). We observed high AP specific activity in MV as compared to EC isolated from chick tibiotarsal growth cartilage, which agrees with the literature (3). The activity of AP was not affected by the dietary lipid treatments, although short chain fatty acids have been reported to alter AP activity in certain biological membranes (20).

It was important to note that chicken cartilage tissues, chondrocytes and MVs all contained large amounts of saturated and monounsaturated fatty acids (>80%) and low levels of n-6 PUFA (<10.2%). In contrast to cartilage lipids, liver polar lipids from these chicks contained

TABLE 5

Fatty Acid Compositions (area %) of Polar Lipids Isolated from Articular Cartilage of Eight-week-old Chicks

Fatty acid	Dietary lipid treatments				Pooled SEM
	SBO	BC	MAC	MEC	
16:0	30.42^a	20.84^c	22.77^b	31.49^a	0.56
16:1	4.03^b	6.74^a	6.24^a	6.76^a	0.31
18:0	19.07^b	20.91^a	17.92^b	13.47^c	0.62
18:1	29.83^b	28.60^b	28.96^b	33.81^a	0.63
18:2n-6	7.44^a	4.67^b	5.14^b	4.79^b	0.33
20:3n-9	1.58^c	2.58^b	3.22^a	1.97^c	0.18
20:4n-6	2.77^a	2.53^a	2.52^a	1.17^b	0.14
20:5n-3	0.21^b	0.11^c	0.15^{bc}	0.93^a	0.03
22:6n-3	0.08^b	0.12^b	0.10^b	0.38^a	0.04
SAT	49.49^a	41.75^c	40.69^c	44.96^b	0.78
MONO	33.86^b	35.34^b	35.20^b	40.57^a	0.71
PUFA	12.09^a	10.01^a	11.13^a	9.25^b	0.38
n-3 PUFA	0.30^b	0.23^b	0.25^b	1.32^a	0.08
n-6 PUFA	10.21^a	7.20^b	7.66^b	5.96^c	0.36
n-3/n-6 Ratio	0.03^b	0.03^b	0.03^b	0.22^a	0.01

^{a,b,c}Mean values (n = 8) within rows having different superscripts are significantly different ($P < 0.05$). For abbreviations see Table 1.

22–41% n-6 PUFA, whereas the saturated and monounsaturated fatty acids comprised 50–60% of the total fatty acids (data not shown). The total amounts of saturated fatty acids and PUFAs in epiphyseal cartilage polar lipids, EC and MV of chicks were affected by the different dietary treatments. However, the amounts of monounsaturated fatty acids (16:1 and 18:1) were higher in polar lipids of epiphyseal cartilage of three-week-old chicks fed BC and MEC and in articular cartilage of eight-week-old chicks fed MEC.

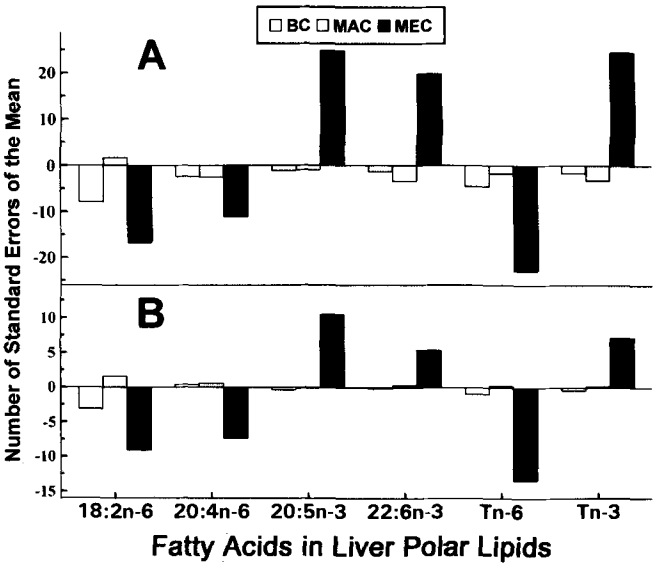

FIG. 1. Polyunsaturated fatty acids (PUFA) in liver polar lipids of chickens at three weeks of age (panel A) and six weeks of age (panel B) presented as standardized differences (STD). The STD for PUFA values were calculated as a difference in treatment mean (butter + corn oil diet; margarine + corn oil diet; or menhaden oil + corn oil diet) from the mean for soybean oil diet (SBO) divided by the pooled SEM (n = 4). An STD value of 2.5 (+ or −) is significantly different ($P < 0.05$) from the SBO treatment.

The fatty acid analyses of articular and epiphyseal cartilages, EC and MV obtained from chicks fed MEC demonstrated that dietary n-3 PUFA are readily incorporated into these tissues and accumulate in neutral lipids of growth cartilage. Interestingly, when chicks were fed a high level of dietary linoleate, 18:2n-6 accumulated in the neutral lipids of chick epiphyseal cartilage. In a study with rats, Lippiello *et al.* (21) found that a menhaden oil diet significantly decreased n-6 PUFA but elevated 20:5n-3 and 22:6n-3 concentrations in articular cartilage.

In the present study, the fatty acid composition of MV was close to that of EC, which is similar to the results reported by Wuthier (5). The low levels of n-6 PUFA in chondrocytes and MV may be related to the involvement of n-6 PUFA in PG biosynthesis in growth cartilage (7).

PGE_2 is known to participate in bone resorption and bone formation (22) and is involved in regulating the activity of cytokines and growth factors in bone (6). Some data (23,24) suggest that altered PUFA metabolism may affect bone and cartilage development by a PG dependent mechanism.

In addition to confirming the presence of Mead acid in growth cartilage, which was first described by Adkisson *et al.* (7), we now quantified high levels of Mead acid in the EC and MV cartilage fractions of chicks on all the dietary treatments.

During early bone development, rapid depletion of arachidonic acid occurs to support eicosanoid biosynthesis (24). The reduction in n-6 PUFA results in lower concentrations of 18:2n-6, which decreases the competition between 18:2n-6 and 18:1n-9 for the Δ6 desaturase and enhances Mead acid formation. Another explanation for the unusual fatty acid composition of cartilage may be that the tissue is sensitive to n-6 PUFA and eicosanoids and, thus, accumulates less n-6 PUFA. Interestingly, our data demonstrate that feeding SBO, a rich source of linoleic acid, had no effect on Mead acid concentrations in growth cartilage, EC or MV. However, we did note a general trend toward a decrease in the concentration of Mead acid in epiphyseal cartilage between three and six weeks, which was independent of the dietary treatment. This agrees well with the findings of Adkisson *et al.* (25), who reported a marked depletion in Mead acid content of phospholipids in human costal and articular cartilages as a function of aging.

Although dietary *trans*-18:1 were reported to be incorporated into liver, lung and abdominal fat pads of chicks (26), as well as into tibiotarsal growth cartilage (27), *trans*-18:1 was not detectable in EC and MV of chicks fed the *trans*-18:1 diet (MAC). Perhaps the amounts of *trans*-18:1 in MAC and BC were insufficient to cause their accumulation in EC as their levels were 14.85 and 2.19%, respectively, compared to the 44.44% used in a previous study (27).

Total n-3 PUFA levels and the n-3/n-6 ratios were the greatest in cartilage, EC and MV of chicks consuming MEC as compared to those fed SBO, BC and MAC. Feeding MEC to chicks lowered the concentrations of 20:4n-6 but increased those of 20:5n-3 and 22:6n-3 in EC and MV.

The rapid enrichment of n-3 PUFA in EC and MV isolated from growth cartilage may imply that the avascularity of cartilage may not be the main reason for the lower n-6 PUFA and higher 20:3n-9 levels as explained by Adkisson *et al.* (7). The effect of enriched n-3 PUFA on the cellular functions of EC and MV in growth cartilage tissue is not presently clear.

The data from our present experiments confirm previous reports that normal cartilage tissues are low in n-6 PUFA and contain 20:3n-9 (Mead acid), and they demonstrate that the concentration of Mead acid appears to be unaffected by the level of dietary essential fatty acids (18:2n-6 and 18:3n-3). *Trans*-18:1 fatty acids did accumulate in epiphyseal cartilage of the proximal tibiotarsus of chicks fed margarine. The data also indicate that n-3 PUFA levels can be increased in cartilage tissue and cells by a diet enriched with n-3 PUFA.

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The Immunosuppressive Substance 2-Chloro-2-deoxyadenosine Modulates Lipoprotein Metabolism in a Murine Macrophage Cell Line (P388 cells)

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A recently developed immunosuppressive substance, 2-chloro-2-deoxyadenosine (2-CdA), was reported to inhibit monocyte functions at low concentration. Because macrophages play a key role in the formation of atherosclerotic plaques, it was of interest to study the effect of 2-CdA on cellular lipid metabolism. For this purpose we have used a macrophage cell line (P388) to perform incubation studies in the presence of acetylated low density lipoprotein (Ac-LDL) and 2-CdA. The addition of 2-CdA, in concentrations ranging from 5–20 nM, induced a dose-dependent decrease in cellular cholesterol content and in the amount of extracellular [^{14}C]oleic acid (OA) incorporated into the cholesteryl ester (CE) fraction. The effect was maximized at 20 nM 2-CdA with an 86% reduction in cholesterol esterification compared to controls ($P < 0.008$). To evaluate the mechanism of interaction of 2-CdA with cellular lipid metabolism, deoxycytidine (dCyt) and 3-methoxybenzamide (3-MOB), substances known to antagonize the effect of 2-CdA in different ways, were co-administered with 2-CdA. dCyt, a competitive inhibitor of dCyt kinase, which catalyzes phosphorylation to the active metabolite, antagonized the effects of 20 nM 2-CdA, producing significantly greater incorporation of extracellular [^{14}C]OA into the CE fraction than in the presence of 2-CdA alone ($P < 0.0086$). Co-incubation with 2-CdA and the poly-ADP-ribose synthetase inhibitor 3-MOB, which is known to render cells resistant to 2-CdA toxicity by preventing cellular nicotinamide adenine dinucleotide (NAD)- and adenosine triphosphase-depletion, also reversed the effect of 2-CdA on lipid accumulation. However, incubation of P388 cells with 20 nM 2-CdA did not result in a decrease in cellular NAD content. As 20 nM 2-CdA showed no effect on intracellular cholesterol synthesis based on measurement by 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, the decrease in cellular cholesterol content and in [^{14}C]OA incorporation seems to be primarily due to an interference with Ac-LDL metabolism.

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Monocyte-derived macrophages play a central role in atherogenesis and constitute the major source of foam cells in atherosclerotic lesions (1–3). Accumulation of cholesteryl esters (CE) in macrophages occurs by the uptake of modified lipoproteins *via* the scavenger receptor pathway (4–6). Several factors have been reported to modulate these receptor activities in human monocytes (3,7), peritoneal murine macrophages (4) and in macrophage cell lines (8). Bacterial endotoxin (9) and lymphocyte conditioned medium (10) were shown to inhibit scavenger receptor activities. Maturation of monocytes to macrophages (11), macrophage conditioned medium and increased plating density all enhance receptor activities (12,13). Based on these results, it has been suggested that the activity of scavenger receptors reflects macrophage differentiation and/or activation. Activation of macrophages was found to be initiated by a lymphokine, interferon γ, which is released by stimulated T-lymphocytes within the atherosclerotic lesion (14). The primed macrophage is susceptible to endotoxins and other stimulatory factors and exhibits increased phagocytotic activity (14,15).

The complex interactions between macrophage activity and the magnitude of acetylated low density lipoprotein (Ac-LDL) degradation is demonstrated by the inhibition reported of scavenger receptor expression by interferon γ (10,14), whereas interferon γ appeared to increase Ac-LDL degradation in another system (16). Dexamethasone, an agent known to depress a number of macrophage inflammatory and immune functions, augments scavenger receptor activity in human monocytes–macrophages (17).

2-Chlorodeoxyadenosine (2-CdA), a new drug with strong immunosuppressive properties (18–20), inhibits macrophage functions like phagocytosis and IL-6 release at low concentrations (20). To investigate a potential effect of 2-CdA on cellular lipid metabolism, we used the murine macrophage line P388, which is considered to be a suitable model for foam cell formation, including studies of the scavenger receptor pathway (8). 2-CdA is a 2-chloro analog of deoxyadenosine, which is resistant to degradation by adenosine deaminase (21). For its biological effects, 2-CdA requires intracellular phosphorylation by deoxycytidine (dCyt) kinase (21). The phosphorylated metabolite chlorodeoxy adenosine triphosphate (cdATP) accumulates in cells rich in this enzyme and poor in 5-nucleotidase (22). It has been suggested that the chlorodeoxyadenosine nucleotides rapidly cause DNA strand breaks in nondividing lymphocytes and monocytes, and that the free ends of DNA activate the enzyme poly(ADP-ribose) polymerase, resulting in NAD and ATP depletion of cells and, consequently, in cell death (23,24). Toxicity of 2-CdA is abolished in lymphocytes and macrophages by dCyt, which acts as a competitive inhibitor of dCyt kinase and thus prevents accumulation of chlorodeoxyadenosine nucleotides (21). The lymphocytotoxic effects of 2-CdA are also blocked by co-incubation of the cells with the ADP

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Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; Ac-LDL, acetylated low denstiy lipoprotein; ADP, adenosine diphosphate; AMP, adenosine–monophosphate; 2-CdA, 2-chloro-2-deoxyadenosine; cdATP, chlorodeoxy adenosine triphosphate; CE, cholesteryl ester; dCyt, deoxycytidine; EDTA, ethylenediaminetetraacetic acid; HMG–CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; 3-MOB, 3-methoxybenzamide; NAD, nicotinamide adenine dinucleotide; OA, oleic acid.

ribosylation inhibitor 3-methoxybenzamide (3-MOB) (25,26).

Because 2-CdA initiates a sequence of well-defined biochemical events in monocytes that can be blocked at enzymatic sites by dCyt and 3-MOB, this drug appeared to be ideal for investigating the effects of immunosuppression on cellular lipid metabolism.

MATERIALS AND METHODS

Lipoprotein preparation. LDL was obtained from the ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood of healthy donors, after overnight fasting, using zonal ultracentrifugation (27). The lipoproteins were dialyzed against saline-EDTA (0.9% NaCl, 1 mM EDTA). Ac-LDL was prepared by acetylating LDL with acetic anhydride as described by Basu *et al.* (28). LDL and Ac-LDL were stored at 4°C under nitrogen and used within one week after preparation. Protein was determined by the method of Lowry *et al.* (29).

Chlorodeoxyadenosine, dCyt and 3-MOB. Chlorodeoxyadenosine was kindly provided by E. Beutler (Scripps Clinic, La Jolla, CA). dCyt was purchased from Sigma (St. Louis, MO). Drugs were stored as dry powder at –20°C and dissolved in sterile 0.9% NaCl prior to use.

Cell lines. The murine macrophage cell line P388 (lymphoid neoplasm of DBA/2 mice treated with 3-methylcholanthrene) was obtained from the Salk Institute Cell Repository (San Diego, CA). The cell culture medium was Hepes-buffered RPMI-1640 supplemented with 1 mM glutamine, 10% heat-inactivated fetal calf serum, 100 μg/mL penicillin and 100 units/mL streptomycin. Cells were maintained in 100-mm dishes in a humidified incubator (5% CO_2) at 37°C.

Cell cultures. For the experiments, 1×10^6 cells/dish (10-mm dishes) were seeded in 1 mL complete medium in triplicate. Cell viability was greater than 90% as assessed by Trypan Blue dye exclusion. Cultures were re-fed 24 h later, when they usually had reached about 70% confluency, with 1 mL of serum-free medium containing the desired concentration of Ac-LDL (10–100 μg/mL) or 25-hydroxycholesterol (Sigma) (25 and 100 μg/mL), to which 2-CdA (5–80 nM) and/or dCyt (10^{-3}–10^{-6} M), 2-CdA and/or 3-MOB (2.5 and 5 mM) and 2-CdA and/or aminophylline (10 and 20 μM) were added, as indicated in the figures.

Time-course studies. In pre-incubation studies, 1×10^6 cells/dish were seeded in 1 mL complete medium in triplicate. After the cells had reached about 70% confluency, cultures were re-fed with complete medium (to which 2-CdA was added), and subsequently incubated for 4, 8 and 24 h. After incubation with 2-CdA, the cells were washed twice with phosphate-buffered saline and then incubated again with 1 mL serum-free medium (RPMI-1640) containing 100 μg/mL Ac-LDL.

To determine whether the 2-CdA antagonizing effect of dCyt is time-dependent, two sets of experiments were used. In one the competitive inhibitor of the 2-CdA activating enzyme dCyt kinase, dCyt and 2-CdA were added simultaneously to the confluent cell cultures in complete medium and incubated for 24 h. Cell monolayers were then washed and incubated with serum-free medium containing Ac-LDL. In the second set of experiments, dCyt was added after treatment of cell cultures with 2-CdA. The cultures were then washed with saline solution and incubated with medium containing Ac-LDL and dCyt.

Cholesterol esterification assay. [^{14}C]Oleic acid (OA) (55 mCi/mmol; Amersham, Arlington Heights, IL), was complexed with bovine serum albumin. Cells were incubated with Ac-LDL and 0.01 M albumin complexed with [^{14}C]OA. After an incubation period of 24 h, cell monolayers were washed and extracted with hexane/isopropanol (3:2, vol/vol) containing 10 μg/mL carrier cholesterol, cholesteryl oleate and [^{3}H]cholesterol to assess recovery. Labeled lipids were taken to dryness, re-dissolved in hexane and separated by thin-layer chromatography on Whatman MK-F microslides using heptane/diethyl ether/acetic acid (75:25:2, by vol) as developing solvent. Lipid fractions were visualized with iodine vapor and scraped into vials for counting (8). Cell monolayers were dissolved in 0.1N NaOH for protein determination according to Lowry *et al.* (29).

Determination of total amounts of cholesterol. Cultures were washed and extracted with hexane/isopropanol (3:2, vol/vol). After solvent removal, total cholesterol, free cholesterol and triglyceride mass were assayed using an enzymatic kit (Cholesterol CHOD-PAP, Triglycerides GPO-PAP; Boehringer Mannheim, Mannheim, Germany).

3-Hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase activity. The rate limiting enzyme in *de novo* cholesterol synthesis, HMG–CoA reductase, was assayed in P388 cells incubated for 24 h with or without 20 nM 2-CdA, according to the method described by Harwood *et al.* (30). HMG–CoA reductase activity in HEPG2 cells served as control in evaluating the assay.

Measurement of intracellular NAD. Cellular NAD content was determined in P388 cells 24 h after plating and subsequent incubation for 4, 8 and 24 h, either with 20 nM 2-CdA and 10^{-3} M dCyt, or alternatively with 20 nM 2-CdA and 2.5 mM 3-MOB. At above time points, samples were also assessed for cell viability. NAD content was quantified by high-performance liquid chromatography (31).

Statistical methods. Data are expressed as mean ± SD for triplicate determinations. The Mann-Whitney U test was used to evaluate differences in the lipid content of the cells treated with and without 2-CdA.

RESULTS

Influence of 2-CdA on [^{14}C]OA incorporation into the CE fraction and on cellular cholesterol and triglyceride levels. The addition of 2-CdA at various concentrations (from 5 to 80 nM) to 1×10^6 P388 cells exposed to different concentrations of Ac-LDL (10–100 μg/mL) resulted in a dose-dependent reduction of the amount of extracellular [^{14}C]OA incorporated into the CE fraction, as compared to control cultures treated with Ac-LDL alone. At 5 nM, 2-CdA caused only a slight reduction in [^{14}C]OA incorporation, but at 10 nM there was a significant decrease compared to controls ($P < 0.009$) as is shown in Figure 1. A further reduction to 86% ($P < 0.008$) was achieved at 20 nM 2-CdA; however, increasing the concentrations to 40 or

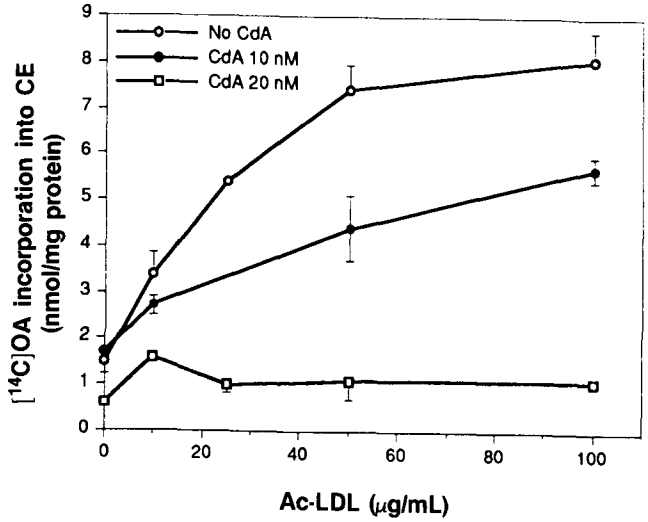


FIG. 1. Cultures grown for 24 h in complete medium were washed and re-fed with serum-free medium supplemented with acetylated low density lipoprotein (Ac-LDL) alone (10, 50 and 100 μg/mL), or Ac-LDL and 2-chloro-2-deoxyadenosine (2-CdA) (5, 10, 20 40 or 80 nM). For cholesteryl ester (CE) determination, 0.1 mM [^{14}C]oleic acid (OA) complexed with bovine serum albumin was added to the cultures. After 24 h, lipids were extracted with hexane/2-propanol (3:2, vol/vol), and the CE content was calculated as described in the Materials and Methods section.

80 nM produced no additional decrease ($P < 0.009$). By contrast, a dose-dependent decrease in total cellular cholesterol content was found with increasing 2-CdA concentrations up to 80 nM (Fig. 2). Concentrations above 80 nM 2-CdA were toxic to P388 cells (data not shown).

Cellular-free cholesterol content did not decrease significantly during 2-CdA incubation (without 2-CdA, 52 ± 9 μg/mg protein; with 2-CdA at 10 nM, 49 ± 10 μg/mg protein, $P < 0.6$; with 2-CdA at 20 nM, 41± 8 μg/mg protein, $P < 0.2$). These results indicate that the decrease in cellular total cholesterol results from a decrease in the amount of cellular CE. Incubation with 2-CdA revealed no significant effect on cellular triglyceride content (without 2-CdA, 127 ± 11 μg/mg protein; with 2-CdA at 10 nM, 115 ± 12 μg/mg protein, $P < 0.4$; with 2-CdA at 20 nM, 108 ± 9 μg/mg protein, $P < 0.07$).

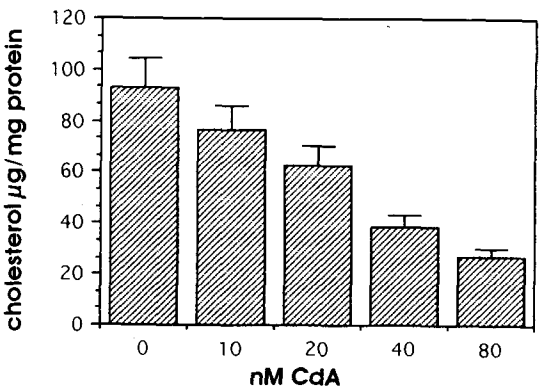


FIG. 2. Cell cultures grown for 24 h in complete medium were re-fed with serum-free medium containing 100 μg/mL Ac-LDL and 2-CdA at concentrations of 10, 20, 40 and 80 nM. After 24 h, lipids were extracted with hexane/2-propanol and the cholesterol content determined enzymatically as described in the Materials and Methods section. Abbreviations as in Figure 1.

When P388 cells were incubated with 25-hydroxycholesterol, which is known to act as a stimulus for CE synthesis by increasing acyl coenzyme A:cholesterol acyltransferase (ACAT) activity (32), the presence of 2-CdA at 20 nM had no influence on extracellular [^{14}C]OA incorporation into the CE fraction (25-hydroxycholesterol, 25 μg/mL: without 2-CdA. 2.3 ± 0.5 nmol/mg protein; with 2-CdA, 2.2 ± 0.3 nmol/mg protein, $P < 0.4$; 25-hydroxycholesterol, 100 μg/mL: without 2-CdA, 3.7 ± 0.5 nmol/mg protein; with 2-CdA, 3.5 ± 0.3 nmol/mg protein, $P < 0.3$).

Cell protein content usually varied by less than 12% within each experiment in the cell cultures exposed to 2-CdA at concentrations between 5 and 80 nM. No significant difference in cell viability was found between cultures without 2-CdA and those incubated with 2-CdA at 20 nM for 24 h (95.7 ± 1.5% and 94.0 ± 1.7% viable cells, respectively). As 20 nM 2-CdA did not affect the viability of P388 cells and produced a plateau for the reducing effect, this dose of 2-CdA was routinely used in the following experiments.

Pre-incubation studies. Previous *in vitro* studies on the effect of 2-CdA on lymphocytes and monocytes have shown that a minimal exposure of 8 h is required for initiation of a sequence of metabolic changes ultimately leading to cell death (22). We performed pre-incubation studies in order to determine the length of time cells need to be exposed to 2-CdA for the optimal effect on lipid metabolism to occur, and to exclude simple interactions of 2-CdA with Ac-LDL or the plasma membrane lipid matrix.

P388 cells were pre-incubated with 2-CdA (20 nM) for 4, 8 and 24 h for this purpose. After incubation, 2-CdA was removed by careful washing, and cells were again incubated with Ac-LDL for 24 h, after which time [^{14}C]OA incorporation into the CE fraction and total cholesterol content were determined. As seen in Table 1, a 24-h exposure to 2-CdA was required for a significant reduction of cellular cholesterol content and cholesterol esterification to occur ($P < 0.008$). Furthermore, a simple 2-CdA/lipoprotein interaction was excluded.

TABLE 1

Effect of Exposure Time of 2-CdA on Cellular Lipid Content[a]

Exposure time	[^{14}C]OA incorporation into CE ± SD (nmol/mg protein)	Total cholesterol ± SD (mg/mg protein)
No CdA	8.1 ± 0.9	96 ± 0.6
4 h	7.7 ± 0.6	88 ± 2.6
8 h	5.3 ± 0.5	78 ± 4.9
24 h	1.1 ± 0.1	67 ± 2.0

[a]Cells (1×10^6 mL) were seeded in complete medium. After 24 h, cells were re-fed with complete medium containing 20 nM 2-chloro-2-deoxyadenosine (2-CdA) for various exposure times (4, 8 and 24 h). After careful washing, serum-free medium containing acetylated low density lipoprotein 100 μg/mL was added. For cholesteryl ester (CE) determination, 0.1 mM [^{14}C]oleic acid (OA) complexed with bovine serum albumin was added to the culture medium. Again after 24 h, lipids were extracted and results calculated as described in the Materials and Methods section.

The role of dCyt kinase. Previous studies have shown that the toxic effects of 2-CdA on monocytes and lymphocytes require phosphorylation catalyzed by dCyt kinase and are prevented by dCyt in cells with low 5′-nucleotidase activity (20,24). In order to examine whether the lipid-lowering effect of 2-CdA also depends on intracellular phosphorylation by dCyt kinase, dCyt, a competitive inhibitor of dCyt kinase, was added simultaneously with both 2-CdA and Ac-LDL to the cell cultures. Up to 10^{-3} M, dCyt alone had no effect on lipid accumulation in foam cells in the presence of 100 μg/mL Ac-LDL; at this concentration [^{14}C]OA incorporation into CE was 7.7 ± 0.6 nmol/mg protein and total cholesterol content was 99 ± 1.0 μg/mg protein. However, when 10^{-3} M dCyt was co-administered with 2-CdA to Ac-LDL-containing cultures, the effect of 2-CdA on cholesterol content and [^{14}C]OA incorporation could be completely reversed (Figs. 3 and 4). [^{14}C]OA incorporation into the CE fraction under these experimental conditions was significantly higher as compared to cultures treated with 2-CdA alone ($P < 0.0086$). This suggests that dCyt kinase plays a role in the effects of 2-CdA on cellular lipid metabolism.

In further experiments dCyt was co-administered with Ac-LDL 24 h after the addition of 2-CdA. the results (Table 2) show that a delayed addition of dCyt could not reverse the lipid-lowering effect of 2-CdA, further indicating that the 2-CdA effect is mediated by a metabolic sequence of events.

Effect of 3-MOB. Previous studies have shown that cellular NAD is preserved by 3-aminobenzamide in 2-CdA treated lymphocytes and monocytes (20). To assess the relationship of ADP-ribosylation to the effect of 2-CdA on cellular cholesterol content and [^{14}C]OA incorporation, P388 cells were treated with 3-MOB, an inhibitor of ribosylation, both alone and in combination. The results show (Figs. 5 and 6) that nontoxic concentrations of 3-MOB administered alone had no effect on lipid metabolism (for 2.5 and 5 mM concentrations, [^{14}C]OA incorporation into CE was 8.7 ± 0.6 and 8.3 ± 0.6 nmol/mg protein, respectively; cholesterol content was 100 ± 1.5 μg/mg protein and 96 ± 0.6 μg/mg protein, respectively), whereas a simultaneous exposure of cells to 3-MOB and 2-CdA prevented the ef-

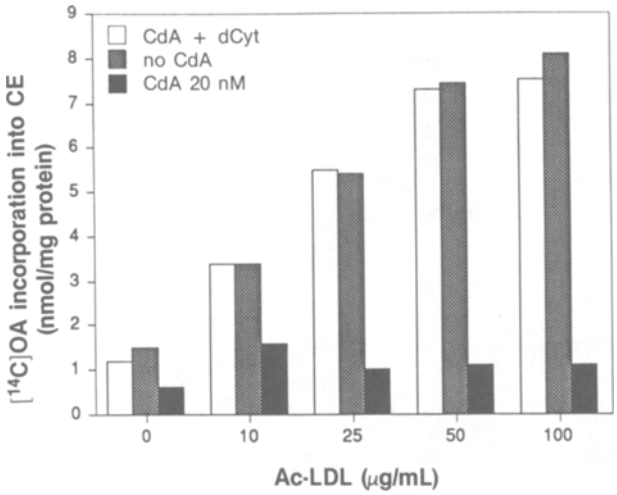


FIG. 3. Cultures grown for 24 h in complete medium were re-fed with serum-free medium containing Ac-LDL alone (10, 25, 50 and 100 μg/mL), Ac-LDL (10, 25, 50 and 100 μg/mL) and 20 nM 2-CdA or Ac-LDL (10, 25, 50 and 100 μg/mL) together with 20 nM 2-CdA and 10^{-3} M deoxycytidine (dCyt). For CE determination, 0.1 mM [^{14}C]OA complexed with bovine serum albumin was added to the cultures; after 24 h, lipids were extracted and results calculated as described in the Materials and Methods section (20 nM 2-CdA vs. 20 nM 2-CdA and 10^{-3} M dCyt; $P < 0.0086$). Abbreviations as in Figure 1.

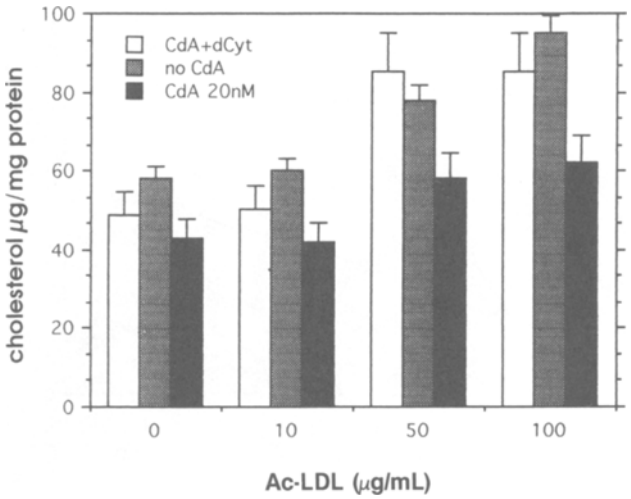


FIG. 4. Cultures were grown for 24 h in complete medium and re-fed with serum-free medium containing Ac-LDL alone (10, 50 and 100 μg/mL), Ac-LDL and 20 nM 2-CdA, or Ac-LDL together with 20 nM 2-CdA and 10^{-3} M dCyt. After 24 h, lipids were extracted and the cholesterol content measured as described in the Materials and Methods section. Abbreviations as in Figures 1 and 3.

TABLE 2

Effects of Delayed dCyt Addition on the Cellular Lipid Content of 2-CdA-Treated Cells[a]

Treatment	[^{14}C]OA incorporation into CE ± SD (nmol/mg protein)	Total cholesterol ± SD (mg/mg protein)
2-CdA	1.1 ± 0.1	67 ± 2.1
2-CdA and dCyt simultaneously	7.3 ± 0.6	85 ± 3.6
2-CdA followed by dCyt	1.7 ± 0.6	72 ± 3.0
dCyt	7.7 ± 0.6	99 ± 1.0

[a]Cells were grown for 24 h in complete medium and re-fed with serum-free medium containing 100 μg/mL acetylated low density lipoprotein (Ac-LDL). 20 nM 2-CdA, 20 nM 2-CdA together with 10^{-3} M deoxycytidine (dCyt), or 10^{-3} M dCyt alone was added to these cultures with Ac-LDL. In an equivalent number of plates, cells were pre-incubated with 20 nM 2-CdA in complete medium for 24 h. After this pre-incubation period, cells were carefully washed and incubated in serum-free medium containing 100 μg/mL Ac-LDL and 10^{-3} M dCyt. For determination of CE, 0.1 mM [^{14}C]OA complexed with bovine serum albumin was added to the cultures. After 24 h lipids were extracted and results calculated as described in the Materials and Methods section. Abbreviations as in Table 1.

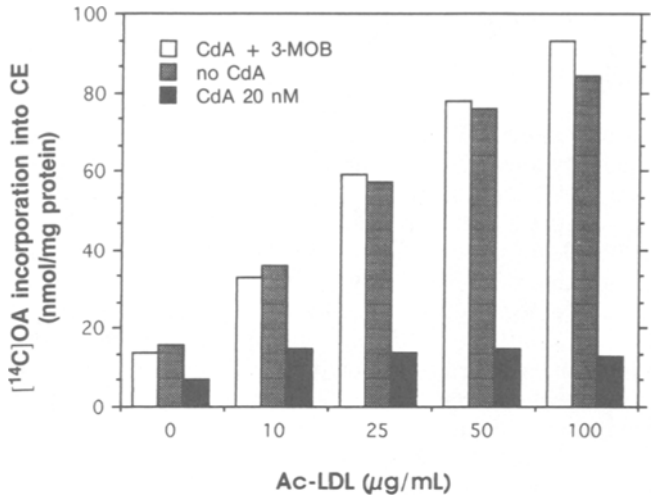


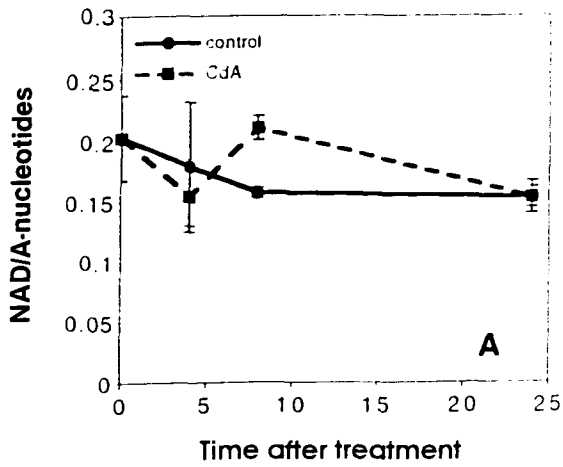
FIG. 5. Cultures were grown in complete medium for 24 h and re-fed with serum-free medium containing Ac-LDL alone (10, 25, 50 and 100 μg/mL), Ac-LDL and 20 nM 2-CdA, or Ac-LDL together with 20 nM 2-CdA and 5 nM 3-methoxybenzamide (3-MOB). For CE determination, 0.1 mM [^{14}C]OA complexed with bovine serum albumin was added to the cultures. After 24 h, lipids were extracted and results calculated as described in the Materials and Methods section. Abbreviations as in Figure 1.

fect of 2-CdA on cellular cholesterol levels and cholesterol esterification.

Effect of aminophylline. To investigate whether the 2-CdA effect depends on intracellular cyclic-AMP levels, which have recently been shown to stimulate LDL receptor activity and hydrolysis of lysosomal cholesterol (33), the xanthine derivative aminophylline (10 and 20 μM) was added to cell cultures incubated with Ac-LDL and 2-CdA. Aminophylline could antagonize the lipid-lowering effect of 2-CdA—the amount of [^{14}C]OA incorporated into the CE fraction increased to 84 and 94% of the values obtained without 2-CdA (10 μM aminophylline, 7.6 ± 0.6 nmol/mg protein; 20 μM aminophylline, 8.3 ± 0.2 nmol/mg protein).

HMG–CoA reductase activity. The addition of 20 nM 2-CdA to P388 cells showed no effect on cellular cholesterol synthesis as measured by HMG–CoA reductase activity (4.8 ± 0.75 nM mevalonate/min/mg protein in 2-CdA treated cells; 4.6 ± 0.2 nM mevalonate/min/mg protein in controls).

Cellular NAD content. In contrast to the previously described decline in cellular NAD content in monocytes treated with high doses of 2-CdA (1 μM) (24), NAD content in P388 cells did not decrease after incubation with



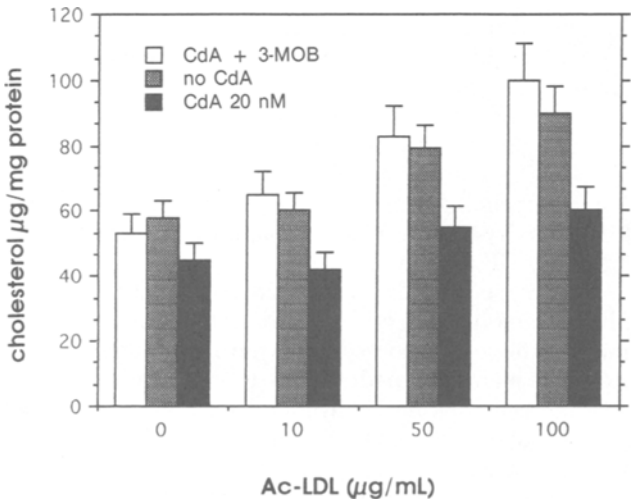


FIG. 6. Cultures were grown for 24 h in complete medium and re-fed with serum-free medium containing Ac-LDL alone (10, 50 and 100 μg/mL), Ac-LDL and 20 nM 2-CdA, or Ac-LDL together with 20 nM 2-CdA and 5 mM 3-MOB. After 24 h, lipids were extracted and total cholesterol was measured as described in the Materials and Methods section. Abbreviations as in Figures 1 and 5.

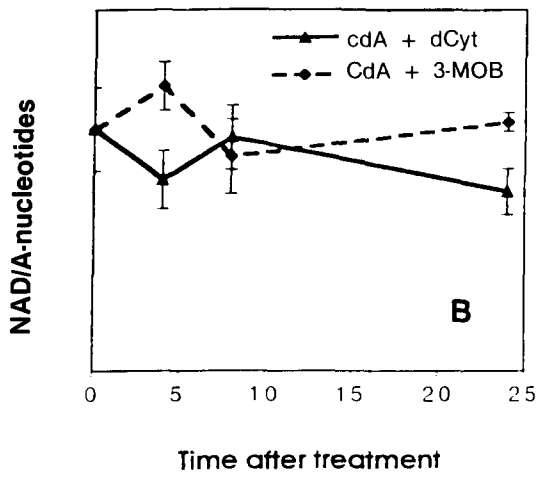


FIG. 7. Cell cultures were plated in complete medium for 24 h. Thereafter, (A) 2-CdA (to a final concentration of 20 nM), and (B) 20 nM 2-CdA and 10^{-3} M dCyt and 20 nM 2-CdA and 2.5 mM 3-MOB were added. After the incubation period indicated, cells were harvested and nucleotide content determined by high-performance liquid chromatography. The values for NAD content per sum of nucleotides are given. Abbreviations as in Figures 1, 3 and 5.

20 nM 2-CdA, as is seen in Figure 7A. The NAD concentrations of the cell cultures without treatment, of those treated with CdA alone, and of those treated with a combination of 2-CdA and dCyt, were similar. Co-incubation of cell cultures with 2-CdA and 3-MOB, an inhibitor of ADP-ribosylation, led to slightly elevated levels of NAD after 4 and 24 h (Fig. 7B).

DISCUSSION

The murine cell line P388 was originally derived from a lymphoid neoplasm of mice treated with 3-methylcholantrene (8). Like monocytes, P388 cells express scavenger receptors, and uptake of Ac-LDL by their scavenger receptor results in foam cell formation.

We now report that 2-CdA, a drug with immunosuppressive properties, modulates lipoprotein metabolism, in that it reduces, in a dose-dependent manner, cholesterol content and incorporation of [^{14}C]OA into the CE fraction of P388 cells incubated with Ac-LDL. Maximal inhibition of up to 86% occurred at a 2-CdA concentration of 20 nM after 24 h. Based on our viability studies and measurement of the total protein content in cultures with and without 2-CdA, the reduced CE content of P388 is not related to increased cell death.

Furthermore, our investigations demonstrate that sequential metabolic changes, similar to those described for normal human monocytes and lymphocytes, must be responsible for the effects of 2-CdA on lipid metabolism in P388 cells. In order to evaluate the mode of action of 2-CdA that is operative, dCyt and 3-MOB, which are known to antagonize the effect of 2-CdA in two different ways, were co-administered with 2-CdA to the cultures.

2-CdA, like deoxypurinenucleosides, enters cells through an efficient transport system and is phosphorylated by deoxycytidine kinase to the corresponding mononucleotide (23). The observed reversal of the effect of 2-CdA by simultaneous addition of dCyt implicates dCyt kinase activity and phosphorylation of 2-CdA to the active metabolite as responsible for the observed modulation of lipid metabolism. A 24-h delay of dCyt addition could not reverse the reduced Ac-LDL metabolism caused by 2-CdA. These results exclude a simple nonspecific interaction of 2-CdA with Ac-LDL or the plasma membrane lipid matrix. Deoxynucleotides can only accumulate in cells rich in deoxycytidine kinase and poor in 5-nucleotidase (22), which seems to be the case in P388 cells.

The nucleotide metabolite of 2-CdA is incorporated into DNA of nondividing monocytes and lymphocytes and rapidly causes DNA strands to break (20,24). The DNA damage is followed by progressive decrease in RNA synthesis and by accelerated consumption of NAD for poly-ADP-ribose synthesis. Ultimately, these biochemical events lead to a decline in ATP levels and cell death.

Based on these observations, it seemed reasonable to speculate that NAD-consumption for poly-ADP-ribose synthesis might also be involved in the altered Ac-LDL metabolism in P388 cells. Our *in vitro* results showed that 3-MOB, an inhibitor of ADP-ribosylation, preserved the cholesterol content and the amount of [^{14}C]OA incorporated into the CE fraction of P388 cells during exposure to 2-CdA. However, the measurement of NAD in P388 cells after a 2-CdA incubation period for 4, 8 and 24 h did not reveal a decline of intracellular NAD levels when compared to untreated cells. This could be explained by the lower amount of DNA strand breakage caused by the significantly lower 2-CdA concentration in our experiments, namely, 20 nM CdA instead of 1 μM CdA (19). Therefore, we suggest that the preservation of cholesterol content and cholesterol esterification in P388 cells by the ribosylation inhibitor 3-MOB is not primarily the result of maintenance of intracellular NAD levels, but might be mostly due to inhibition of ADP-ribosylation of chromosomal proteins. Poly-ADP-ribosylation of specific groups of chromosomal proteins has been observed during DNA repair, gene expression and cell differentiation (34).

Cyclic AMP-elevating agents have been reported to stimulate LDL receptor protein synthesis, as well as hydrolysis of lysosomal CE (33). The xanthine amine derivative aminophylline effectively antagonized the effect of 2-CdA on cellular CE synthesis. These results could indicate an association between the CE-lowering effect of 2-CdA and intracellular cyclic AMP levels.

The low 2-CdA concentrations used in our experiments did not reduce the viability of the P388 cells. This is in agreement with previous studies, which demonstrated that these low 2-CdA concentrations inhibit monocyte phagocytosis and reduce interleukin-6 release, but only higher 2-CdA concentrations led to a dose-dependent loss of monocyte viability (20). Our data provide evidence for another effect of low 2-CdA concentration on cell function which results in reduced cellular cholesterol contents and [^{14}C]OA incorporation into the CE fraction. As 2-CdA did not show an effect on intracellular cholesterol synthesis, the modulation in lipid metabolism seems to depend primarily on an altered Ac-LDL metabolism. Co-incubation of 2-CdA with 25-hydroxycholesterol, a substance known to increase ACAT activity (32), revealed no significant effect on the amount of extracellular [^{14}C]OA incorporated into the CE fraction. The effect of 2-CdA on Ac-LDL metabolism might be due to a decreased scavenger receptor activity, a decreased rate of lipoprotein internalization, a change in the intracellular pathway after internalization of Ac-LDL, or might be due to increased retroendocytosis (3,7,28). However, co-incubation studies of 2-CdA with 25-hydroxycholesterol, a substance known to increase ACAT activity (32), revealed no significant effect of 2-CdA on cellular CE synthesis.

An increase in Ac-LDL degradation during incubation of human monocytes with the immunosuppressant dexamethasone was reported elsewhere (17). The effects of dexamethasone are mediated by the intracellular glucocorticoid receptor, and involve increased cellular RNA levels and protein synthesis. Whereas stimulation of cellular protein synthesis could explain why dexamethasone augments scavenger receptor activity and thus Ac-LDL degradation, 2-CdA might reduce cellular lipid content by causing DNA strand breakage, leading to decreased RNA and protein synthesis (22).

The immunosuppressive 2-CdA, which, at a low concentration, affects several monocyte functions, including cellular lipid metabolism, could possibly provide a tool

to analyze various steps in the development of the atherosclerotic plaque, as the action of 2-CdA on lipid metabolism can be abolished by the competitive inhibitor dCyt and by the ADP ribosylation inhibitor 3-MOB. Further investigations will be necessary to define the exact mechanism of 2-CdA on cellular lipoprotein metabolism.

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Dietary Polyunsaturated Fat Decreases Interaction Between Low Density Lipoproteins and Arterial Proteoglycans

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Polyunsaturated dietary fat (n-3 and n-6) results in less atherosclerosis in monkeys compared to lard (Parks, J.S., Kaduck-Sawyer, J., Bullock, B.C., and Rudel, L.L., *Arteriosclerosis 10,* 1102–1112; Rudel, L.L., Parks, J.S., Johnson, F.L., and Babiak, J., *J. Lipid Res. 27,* 465–474, 1986). We hypothesized that this was due, in part, to a decreased reactivity of low density lipoproteins (LDL) with arterial proteoglycans (PG). To test this hypothesis, cynomolgus monkeys were fed diets containing lard, safflower oil (n-6 polyunsaturated; Poly), menhanden fish oil (FO), or oleic acid-rich safflower oil (oleinate; Mono) for 14 mon, and plasma LDL were isolated and characterized. Several properties of LDL thought to be important in the interaction of LDL with arterial PG were measured including LDL particle size, chemical composition, sialic acid content, density distribution, apolipoprotein E (apoE) content and cholesteryl ester transition temperature. Plasma LDL cholesterol concentrations (mg/dL) after 14 mon of diet consumption averaged (mean ± SEM): FO (366 ± 45), Lard (352 ± 27), Poly (279 ± 24), and Mono (230 ± 43). The composition of LDL was similar among diet groups except that FO LDL were relatively depleted of cholesteryl ester and enriched in protein and were smaller in size. LDL sialic acid content was similar among diet groups (4.5–5.0 μg/mg LDL protein). The LDL apoE/B molar ratio, a measure of the apoE content per LDL particle averaged: Mono (3.0 ± 1.0), Poly (2.0 ± 0.1), Lard (1.8 ± 0.5), and FO (1.0 ± 0.2). The FO group had a lower proportion (13%) of the apoE enriched d = 1.015–1.025 g/mL subfraction of LDL than did the other diet groups (31–45%). The transition temperature of the LDL cholesteryl esters was below body temperature for the FO and Poly groups (36°C) and above for the Lard and Mono groups (40–44°C). The percentage of LDL cholesterol that formed insoluble complexes with arterial chondroitin sulfate PG averaged: Mono (29 ± 4%), Lard (18 ± 3%), Poly (14 ± 3%), and FO (7 ± 2%). Among all diet groups, there was a significant positive correlation (r = 0.54) between LDL–PG complex formation and LDL apoE/B molar ratio. We conclude that dietary FO and Poly result in LDL that are less reactive with arterial PG compared to Lard or Mono fats. While FO appears to decrease PG binding by decreasing the apoE content and amount of the largest LDL subfraction, Poly fat appears to affect LDL–PG interactions by other mechanisms. Decreased LDL–PG interactions may lead to decreased atherosclerosis in animals fed polyunsaturated dietary fat. *Lipids 29,* 635–641 (1994).

Isocaloric substitution of fish oil (FO) for saturated fat in the diet of nonhuman primates results in less coronary artery (1) and abdominal aorta (2) atherosclerosis. In general, diets enriched in n-3 fatty acids result in lower low density lipoprotein (LDL) cholesterol concentrations in nonhuman primates (1,2), but in some cases, LDL cholesterol concentrations are unchanged compared to more saturated fat dietary groups (3). However, dietary FO in African green and cynomolgus monkeys consistently results in LDL that are smaller, contain less cholesteryl ester (CE) and more polyunsaturated CE species, and have a lower CE liquid crystalline-to-liquid transition temperature (3–5). Additional studies in cynomolgus monkeys have demonstrated a relative depletion of LDL apolipoprotein E (apoE) and an increased proportion of denser LDL subfractions in animals fed FO vs. Lard-containing diets (6). These studies illustrate that significant changes in LDL particle composition can occur with n-3 fatty acid-rich diets with little effect on plasma LDL cholesterol concentrations and that these changes in LDL particle composition may affect progression of atherosclerosis.

Several studies have shown a strong positive correlation between coronary artery or abdominal aorta atherosclerosis and LDL size, CE fatty acid composition and apoE content in nonhuman primates (1,7,8). One of the first steps of arterial lipid accumulation in atherosclerosis is thought to involve trapping of LDL in the arterial wall, presumably through interaction with arterial proteoglycans (PG) (9–11). PG are electronegative macromolecular glycoconjugates composed of sulfated glycosaminoglycan chains covalently linked to a protein core and are ubiquitous components of the extracellular matrix (12). In the presence of divalent cations, arterial PG bind to LDL, presumably through the interaction of the sulfated glycosaminoglycan chains of the PG with regions of apoB protein that bind to the glycosaminoglycan heparin (13). ApoE also contains two regions that bind heparin (14). Once formed, LDL–PG complexes formed *in vitro* or isolated from atherosclerotic arteries are capable of stimulating CE accumulation in cultured macrophages (15–17).

To determine whether any of the atherogenic properties of LDL that correlate with atherosclerosis development also influence LDL binding to arterial PG, we studied the interaction of LDL with arterial chondroitin sulfate proteoglycans. LDL from animals fed an FO diet were smaller, more dense and contained less apoE compared to

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoE, apolipoprotein E; CE, cholesteryl ester(s); EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FO, fish oil; HDL, high density lipoprotein(s); HDL-C, high density lipoprotein-cholesterol; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein(s); Mono, monounsaturated fat (i.e., oleinate); PG, proteoglycan(s); Poly, polyunsaturated fat (i.e., safflower oil); TPC, total plasma cholesterol.

those from animals fed a Lard diet (3,6). FO LDL also bound less avidly to the arterial PG *in vitro* (6,18) consistent with the decreased atherosclerosis development in FO-fed nonhuman primates (1,2). In addition, there was a positive correlation between LDL–PG complex formation and the apoE content of the d = 1.015–1.025 g/mL subfraction (6). However, it is unclear whether the decreased PG interaction with FO LDL was due to a specific effect of n-3 fatty acids, *per se,* or whether a similar result would have been observed with dietary polyunsaturated fat enriched in n-6 fatty acids.

The purpose of the present study was to compare and contrast the effects of dietary n-3 and n-6 polyunsaturated fat relative to more saturated dietary fats (Lard and oleinate) on LDL composition, density subfraction heterogeneity, and PG binding. Based on our previous study (6), we hypothesized that both n-6 as well as n-3 dietary polyunsaturated fat would decrease the amount of LDL apoE, resulting in less PG binding compared to more saturated dietary fats.

MATERIALS AND METHODS

Experimental design. A subset of 25 feral adult male cynomolgus monkeys *(Macaca fascicularis)* from Indonesia were chosen for these studies from a larger group of 52 monkeys. The monkeys were purchased and placed in quarantine for 14 wk, during which time they received a monkey chow diet (Ralston Purina Co., St. Louis, MO). The animals were then fed for 12 wk an atherogenic challenge diet containing 40% of calories from fat and 0.45 mg/kcal cholesterol to assess the dietary responsiveness of each animal. After the challenge phase, the animals were fed monkey chow for a 14-week "washout" period to allow plasma lipids to return to baseline concentrations. This was followed by an experimental phase in which animals were assigned to one of four diet groups [Lard, monounsaturated fat (mono), polyunsaturated fat (poly) or FO] based on the challenge phase data so that each group had equivalent total plasma cholesterol (TPC), high density lipoprotein (HDL)-cholesterol (HDL-C) and body weight values. For this study, a subgroup of 6–7 animals from each diet group was chosen so that TPC and HDL-C concentrations of the subgroup were not significantly different from the parent group for each of the dietary fats.

Diets. The experimental diets contained 35% of calories as fat and 0.40 mg cholesterol/kcal. The four experimental fats included Lard, oleic acid-rich safflower oil (oleinate), safflower oil (n-6 polyunsaturated) and menhaden fish oil (n-3 polyunsaturated). Detailed compositions of the diets will appear in a separate publication (Thornburg, J.T., Parks, J.S., and Rudel, L.L., manuscript in preparation). The oleinate oil was provided by the Institute of Shortening and Edible Oils, Inc. (Washington, D.C.). Fish oil from the Southeast Fisheries Center (Charleston, SC) was isocalorically substituted for half of the Lard calories while the parent fat was used for the other three diets. β-Sitosterol and cholesterol content were made equivalent among the four diets. The α- and δ-tocopherols and Tenox 20A (*tert.*-butylhydroquinone; Eastern Chemical Products, Kingsport, TN) were added to the diets to balance the amounts contained in the FO diet.

LDL isolation and chemical characterization. Blood samples were taken from the animals after at least 14 mon of experimental diet consumption and following an overnight fast. Following the administration of ketamine hydrochloride (10 mg/kg), 25 mL of blood was drawn from the femoral vein into chilled tubes (4°C) containing 0.1% ethylenediaminetetraacetic acid (EDTA) and 0.02% NaN_3 (final concentrations) at pH 7.4. Plasma was isolated from each sample by low-speed centrifugation. Lipoproteins were isolated from plasma by ultracentrifugation and were fractionated by size using a Superose 6B high-performance liquid chromatography column as described previously (3). Cholesterol distribution of the isolated lipoproteins was assayed using enzymatic methods (19). LDL sialic acid content was determined by the method of Codington *et al.* (20) and the results were normalized to mg LDL protein. The thiobarbituric acid reactive substances assay was used to determine the extent of lipid peroxidation among LDL samples from the four diet groups (21). LDL samples were stored at 4°C under an argon atmosphere.

Density gradient subfractionation of LDL. Isolated LDL samples were subfractionated by density gradient centrifugation using 5 mg of LDL protein. Discontinuous salt gradients were set up using 39-mL quick-seal centrifuge tubes by first adding 10 mL of a d = 1.006 g/mL solution and then successively underlayering 19 mL of a d = 1.030 g/mL solution (including the LDL sample) and 10 mL of a d = 1.060 g/mL solution. LDL was subfractionated using a VTi-50 vertical rotor at 242,000 × *g* at 15°C for 6 h (22). Individual tubes were pooled to give three density fractions of LDL: d = 1.015–1.025 g/mL, d = 1.025–1.035 g/mL and d = 1.035–1.045 g/mL. These subfractions were dialyzed against 0.9% NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4, and concentrated to 2 mL for chemical analyses. LDL and LDL subfraction chemical compositions were determined as described previously (23). Plasma LDL and LDL subfraction apolipoproteins E and B_{100} were assayed using an enzyme-linked immunosorbent (ELISA) assay (24,25).

LDL–PG binding studies. LDL binding to isolated arterial chondroitin sulfate PG was investigated as described previously (18). Briefly, 100 μg of LDL cholesterol was incubated with 1 μg PG (measured as hexuronic acid) in buffer containing 5 mM Tris, 6 mM KCl, 15 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.2, at 26°C for 30 min. The insoluble LDL–PG complexes that formed were isolated by low-speed centrifugation. The supernatant was removed, the pellet was resuspended and LDL cholesterol was measured enzymatically (19). Data are presented as percentage of total LDL cholesterol present as insoluble PG complex.

Data analysis. Data are presented as mean ± standard error of the mean. Statistical analyses were done using the Statview™ SE+ program for the Macintosh computer. One-way analysis of variance was used to detect a main effect of diet. Analysis of covariance was used to adjust PG binding data for differences in LDL cholesterol to apoB ratio and LDL apoE content. When a significant effect was

disclosed, Fisher's least significant difference test was used to compare individual diet group differences.

RESULTS

Total plasma and lipoprotein cholesterol concentrations for animals consuming the four experimental diets are shown in Table 1. TPC and very low density lipoprotein and intermediate-size LDL cholesterol concentrations were not significantly different among the dietary groups. Average LDL cholesterol concentrations for animals fed FO and Lard diets were higher than those consuming the Mono or Poly diets. The HDL cholesterol concentrations were significantly lower for the FO and Poly groups compared to the Mono and Lard groups. The animals consuming the FO diet had significantly smaller LDL particles, measured as LDL molecular weight, than those consuming the other three experimental diets. The LDL cholesterol/apoB ratio, an independent estimate of LDL size, followed the same trend as LDL molecular weight among diet groups.

Because of the observed differences in LDL cholesterol concentration and LDL size among the experimental diet groups, LDL subfraction heterogeneity was investigated by density gradient ultracentrifugation. Representative density gradient profiles for one animal from each diet group are shown in Figure 1. The LDL from the animal fed the FO diet was more dense and distributed predominantly in the lower half of the gradient while the LDL from animals fed the Lard, Mono, or Poly diets were less dense and distributed predominantly in the upper half of the gradient. LDL density heterogeneity was apparent for all animals regardless of diet group. Three density fractions from the LDL gradients were pooled for subsequent analysis.

The LDL apoB and cholesterol subfraction distribution for the three density cuts is shown in Table 2. As suggested from the density gradient profiles in Figure 1, the proportion of apoB in the d = 1.015–1.025 g/mL subfraction of LDL for the FO group was less than one-third that of the other three diet groups ($P < 0.0028$). There was a correspondingly higher proportion of apoB in the d = 1.035–1.045 d/mL subfraction for the FO group compared to the other three diet groups ($P < 0.016$). LDL cholesterol density distribution followed similar trends as LDL apoB.

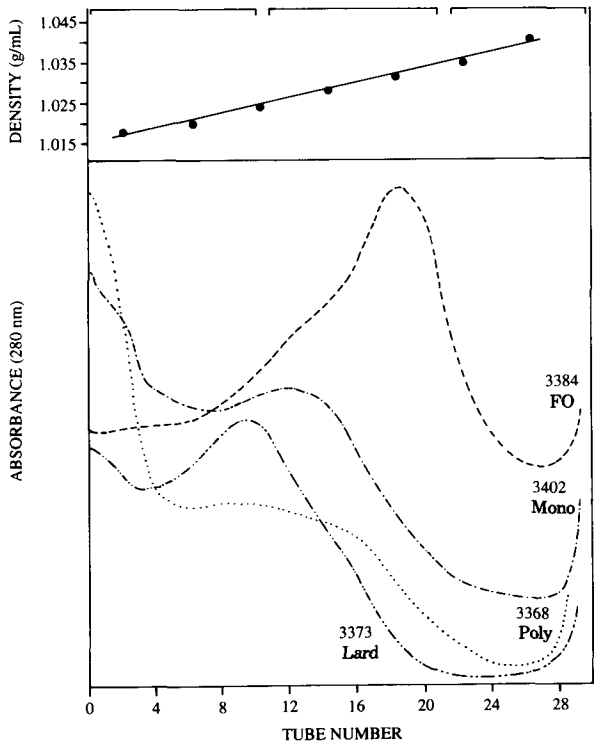

FIG. 1. Density gradient profile of low density lipoprotein (LDL) isolated from plasma by ultracentrifugation and column chromatography. Profiles shown represent one animal from each diet group that had an LDL cholesterol concentration near the group mean. Brackets indicate where subfractions were pooled for subsequent analyses. FO, fish oil; Mono, monounsaturated fat (i.e., oleinate); Poly, polyunsaturated fat (i.e., safflower oil).

Previous studies have shown that much of the apoE in the plasma of cynomolgus monkeys fed atherogenic diets is bound to LDL particles (7,26). To determine the effect of dietary fat type on the LDL subfraction distribution of apoE, the amount of apoE and apoB among the three LDL density subfractions was quantified by ELISA. The values

TABLE 1

Total Plasma and Lipoprotein Cholesterol Concentrations[a,b]

Diet	(mg/dL) TPC	VLDL + ILDL	LDL	HDL	LDL MW (g/μmol)	LDL TC/apoB ratio
Lard (n = 7)	460 ± 35	61 ± 23	352[a] ± 27	28[a] ± 2	4.04[a] ± 0.2	3.39[a] ± 0.52
Mono (n = 6)	316 ± 52	61 ± 15	230[b] ± 43	25[a] ± 3	4.40[a] ± 0.3	4.44[b] ± 0.91
Poly (n = 6)	354 ± 24	59 ± 8	279[a,b] ± 24	16[b] ± 2	4.27[a] ± 0.3	3.83[b] ± 0.16
FO (n = 6)	426 ± 49	47 ± 9	366[a] ± 45	18[b] ± 2	3.11[b] ± 0.1	2.03[a] ± 0.25
P Value	NS	NS	0.043	0.001	0.003	0.037

[a,b]All values represent the means ± SEM. Means with unlike symbols are significantly different ($P < 0.05$) as determined by analysis of variance and Fisher's least significant difference test. Plasma was obtained from animals after 14 mon of experimental diet consumption. TPC, total plasma cholesterol; VLDL, very low density lipoprotein; ILDL, intermediate low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TC, total cholesterol; LDL MW, LDL molecular weight; Mono, monounsaturated fat (i.e., oleic acid-rich safflower oil); Poly, polyunsaturated fat (i.e., safflower oil); FO, fish oil; NS, not significantly different.

TABLE 2

Percentage of ApoB and Cholesterol Distribution in LDL Subfractions[a,b]

Diet group	LDL Density (g/mL) d = 1.015–1.025	d = 1.025–1.035	d = 1.035–1.045
	% apoB distribution		
Lard (n = 7)	44.8 ± 5.2[a]	51.6 ± 4.5	3.7 ± 1.1[a]
Mono (n = 6)	31.1 ± 4.8[a]	61.8 ± 3.4	7.1 ± 1.6[a]
Poly (n = 6)	38.7 ± 8.0[a]	53.8 ± 7.0	7.4 ± 2.8[a]
FO (n = 6)	12.6 ± 2.6[b]	61.4 ± 8.3	26.0 ± 9.5[b]
P Value	0.0028	NS	0.016
	% cholesterol distribution		
Lard (n = 7)	62.7 ± 4.0[a]	35.0 ± 3.7[a]	2.3 ± 0.5
Mono (n = 6)	52.7 ± 5.0[a]	42.9 ± 4.4[a,b]	4.5 ± 0.7
Poly (n = 6)	57.7 ± 6.4[a]	38.8 ± 5.7[a]	3.6 ± 1.0
FO (n = 6)	24.5 ± 5.9[b]	57.7 ± 6.4[b]	18.1 ± 9.4
P Value	0.0002	0.026	NS

[a,b]See footnote to Table 1 for details and other abbreviations; apoB, apolipoprotein B.

were then converted to an apoE/B molar ratio, a measure of apoE per LDL particle, since each LDL particle contains only one molecule of apoB. The data are shown in Table 3. The average E/B molar ratio was ordered: Mono (3.0 ± 0.9) > Poly (2.0 ± 0.1) > Lard (1.8 ± 0.5) > FO (1.0 ± 0.2). The effect of diet was of borderline significance ($P = 0.059$). The d = 1.015–1.025 g/mL subfraction had a significantly higher apoE/B molar ratio for the Mono and Poly diet groups compared to the FO and Lard diet groups ($P = 0.0306$). The apoE/B molar ratio was not significantly different among diet groups for the d = 1.025–1.035 g/mL or the d = 1.035–1.045 g/mL subfraction.

The chemical composition of the LDL subfractions and total LDL for the four diet groups is shown in Table 4. LDL from the FO group contained relatively more protein and less CE compared to the other three diet groups, consistent with the smaller size of the FO LDL (i.e., decreased LDL molecular weight and LDL cholesterol/apoB ratio; Table 1). A similar trend was also observed for the d = 1.015–1.025 g/mL subfraction, although the compositional differences did not reach statistical significance. Overall, the compositional differences of LDL subfractions among diet groups were small.

To determine whether the type of dietary fat had modified the LDL CE liquid crystalline-to-liquid transition temperature, a differential scanning calorimetric study was performed; the results are shown in Table 5. The mean transition temperatures for LDL were ordered: Lard > Mono > Poly = FO. The mean transition temperature for both of the polyunsaturated diet groups (i.e., Poly and FO) was below body temperature, whereas it was above body temperature for the Lard and Mono groups. These data confirmed that the different types of dietary fat had altered the CE fatty acid composition of LDL consistent with results of previous studies (3,4).

The results of the PG binding assay are shown in Figure 2. Each point represents data from an individual animal. On average, the percentage of LDL forming an insoluble complex with PG was ordered: Mono (29 ± 4%) > Lard (19 ± 3%) > Poly (14 ± 3%) > FO (7 ± 2%). The mean LDL–PG binding for the FO group was significantly lower than means for the other three diet groups ($P < 0.05$). If data were pooled and analyzed as polyunsaturated (n-3 plus n-6) vs. Lard plus Mono, there was also a significantly lower percentage of LDL cholesterol complexed with PG for the combined polyunsaturated group ($P <$

TABLE 3

LDL Subfraction ApoE/B Molar Ratio[a,b]

Diet	Total LDL	LDL Subfraction (g/mL) d = 1.015–1.025	d = 1.025–1.035	d = 1.035–1.045
Lard (n = 7)	1.8 ± 0.5	2.4 ± 0.3[a]	1.0 ± 0.2	3.5 ± 0.2
Mono (n = 6)	3.0 ± 0.9	4.6 ± 0.9[b]	1.0 ± 0.3	2.0 ± 0.8
Poly (n = 6)	2.0 ± 0.1	4.7 ± 0.9[b]	1.1 ± 0.1	1.5 ± 0.5
FO (n = 6)	1.0 ± 0.2	2.7 ± 0.6[a]	1.3 ± 0.3	0.7 ± 0.2
P Value	0.059	0.0306	NS	NS

[a,b]Apo E and B were measured on individual LDL subfractions by ELISA and converted to a molar ratio using 35,000 and 512,000 as molecular weights for apoE and apoB, respectively. See footnote to Table 1 for further details and abbreviations.

TABLE 4

Chemical Composition of Total LDL and LDL Subfractions[a,b,c]

Density	Diet group	n	% Composition Pro	PL	TG	FC	CE
1.015–1.025	Lard	7	13 ± 0.3	20 ± 0.5	1.4 ± 0.2	8 ± 0.2[a]	57 ± 0.9
	Mono	6	13 ± 0.7	19 ± 0.5	0.8 ± 0.1	9 ± 0.4[a]	58 ± 0.6
	Poly	6	13 ± 0.3	18 ± 0.6	1.0 ± 0.2	9 ± 0.4[a]	59 ± 0.7
	FO	6	14 ± 1.5	21 ± 1.3	1.1 ± 0.2	15 ± 3.3[b]	49 ± 5.3
	P Value		NS	NS	NS	0.023	NS
1.025–1.035	Lard	7	19 ± 0.7	22 ± 0.5[a]	1.2 ± 0.1[a,b]	10 ± 0.4	49 ± 1.2
	Mono	6	19 ± 0.6	19 ± 0.4[b]	0.7 ± 0.2[a]	9 ± 0.7	52 ± 0.8
	Poly	6	20 ± 0.5	18 ± 0.6[b]	0.8 ± 0.2[a]	9 ± 0.4	52 ± 0.8
	FO	6	18 ± 0.5	21 ± 0.6[a]	1.5 ± 0.2[b]	9 ± 0.8	50 ± 1.0
	P Value		NS	0.0004	0.012	NS	NS
1.035–1.045	Lard	7	28 ± 1.3	22 ± 1.0[a]	0.7 ± 0.2	20 ± 3.8	30 ± 4.9
	Mono	6	27 ± 1.0	19 ± 0.6[b,c]	0.7 ± 0.1	13 ± 1.7	41 ± 1.6
	Poly	6	29 ± 1.2	18 ± 0.4[b]	1.1 ± 0.2	20 ± 5.2	32 ± 6.0
	FO	6	26 ± 2.7	21 ± 0.9[a,c]	1.3 ± 0.2	18 ± 4.7	33 ± 8.1
	P Value		NS	0.012	NS	NS	NS
Total LDL	Lard	7	16 ± 1.1[a]	19 ± 1.3	1.3 ± 0.2[a]	8 ± 0.4	56 ± 1.2[a]
	Mono	6	16 ± 1.9[a]	19 ± 0.3	0.8 ± 0.1[b]	8 ± 0.2	56 ± 0.9[a]
	Poly	6	17 ± 0.8[a]	18 ± 0.5	0.8 ± 0.2[b]	8 ± 0.2	57 ± 0.5[a]
	FO	6	19 ± 1.3[b]	20 ± 0.3	1.4 ± 0.1[a]	7 ± 0.3	52 ± 0.8[b]
	P Value		NS	NS	0.018	NS	0.005

[a,b,c]All values represent the means ± SEM. Means with unlike symbols are significantly different ($P < 0.05$) as determined by analysis of variance and Fisher's *post hoc* least significant difference test. PL, phospholipid; TG, triglyceride; FC, free cholesterol; CE, cholesteryl ester. For other abbreviations, see footnote to Table 1.

0.002; Student's *t*-test). The same statistically significant trend in LDL binding to PG was observed after analysis of covariance to adjust for differences in LDL cholesterol/apoB ratio among diet groups.

Our previous study suggested that part of the difference in LDL–PG complex formation between Lard and FO LDL could be explained by the decreased apoE content of FO LDL (6). To determine whether LDL apoE content influenced diet-induced difference in LDL–PG complex formation, the average LDL apoE/B molar ratio was plotted against LDL–PG complex formation (Fig. 3). There was a positive relationship between LDL apoE content and the ability of LDL to form insoluble complexes with PG. The correlation coefficient (r) for the same plot of individual animal data was 0.54 ($P < 0.05$). However, analysis of covariance revealed that significant differences in LDL–PG binding remained after adjusting for differences in LDL apoE/B molar ratio among diet groups.

Previous studies suggested an inverse relationship between LDL sialic acid content and LDL–PG binding. To determine whether differences in sialic acid content among diet groups was related to the differences in LDL–PG complex formation, the sialic acid content of plasma LDL was measured (Table 6). The sialic acid content of the plasma LDL averaged 4.5–5.0 μg/mg LDL protein and was not significantly different among diet groups.

The thiobarbituric acid reactive substances assay was used to determine whether lipid peroxidation was different among LDL samples from the four diet groups. In a subset of three animals from each diet group, there was

TABLE 5

Transition Temperature of Cynomolgus Monkey LDL[a,b,c]

Diet group	Transition temperature (°C)
Lard (n = 7)	44 ± 1.3[a]
Mono (n = 6)	40 ± 0.6[b]
Poly (n = 6)	36 ± 0.6[c]
FO (n = 6)	36 ± 0.9[c]
P Value	0.0001

[a,b,c]See footnote to Table 1 for details and abbreviations.

TABLE 6

Sialic Acid Content of Cynomolgus Monkey LDL[a]

Diet group	μg sialic acid/mg LDL protein
Lard (n = 6)	4.41 ± 0.32
Mono (n = 5)	4.54 ± 0.45
Poly (n = 6)	4.99 ± 0.15
FO (n = 6)	4.97 ± 0.24
P Value	NS

[a]All values represent the means ± SEM. For abbreviations, see footnote to Table 1.

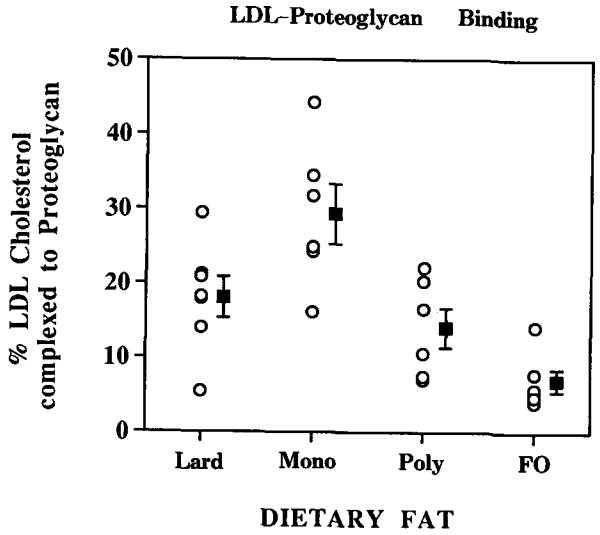

FIG. 2. Formation of particulate complexes between arterial chrondroitin sulfate proteoglycans (PG) (1 μg as hexuronate) and plasma LDL (100 μg as cholesterol). Plasma LDL were isolated from the plasma of cynomolgus monkeys in the four diet groups. Incubations of LDL and PG were performed in 5 mM Tris, 6 mM KCl, 15 mM $CaCl_2$ and 1.5 mM $MgSO_4$ (pH 7.2) for 30 min at 26°C. Formation of particulate PG–LDL complexes was measured as cholesterol in a 1,500 × *g* pellet, which is represented as the percentage of total LDL cholesterol in the incubation. Each open circle represents data from an individual animal; the closed squares represent the mean ± SEM for each diet group. See Figure 1 for other abbreviations.

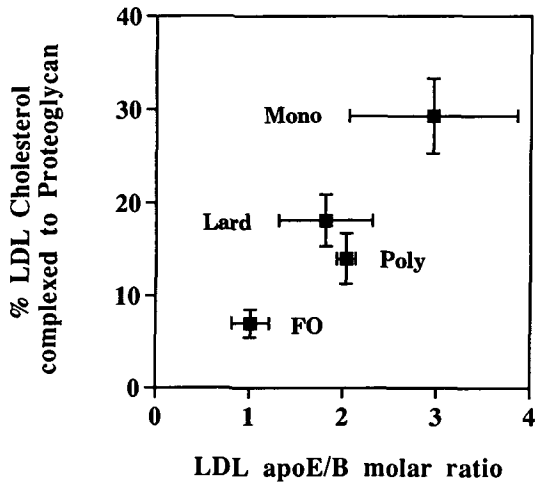

FIG. 3. A plot of LDL apoE/B molar ratio, determined by enzyme-linked immunosorbent assay vs. LDL–PG complex formation assayed as described in Figure 2 legend. Symbols represent the mean ± SEM for each diet group; apoE/B, apolipoprotein E and B. See Figures 1 and 2 for other abbreviations.

<1.1 nmol thiobarbituric acid reactive substances/mg LDL cholesterol, and there was no significant difference, by analysis of variance, among the diet groups.

DISCUSSION

Previous studies have demonstrated that isocaloric substitution of polyunsaturated fat for saturated fat resulted in less coronary artery and aortic atherosclerosis in several nonhuman primate species (1,2,8,27). This has been observed regardless of whether the polyunsaturated dietary fat was enriched in n-6 or n-3 fatty acids. However, the exact mechanism for the reduced atherogenicity of polyunsaturated fat is unknown and is likely to be multifactorial. Since LDL and PG have been co-localized in atherosclerotic lesions of human beings and experimental animals (9–11) and LDL–PG complexes can lead to CE accumulation in macrophages *in vitro* (15,16), we hypothesized that one mechanism by which polyunsaturated dietary fat diminishes atherosclerosis progression might involve a decrease in LDL–PG interaction.

The results of the present study support this hypothesis. FO LDL was significantly less reactive with PG than LDL from the other three diet groups. Poly LDL was significantly less reactive with PG than Mono LDL and, on average, was less reactive than Lard LDL (Fig. 2). Since all other experimental variables except the source of LDL were constant, the data suggest that one or more of the properties of LDL important in the interaction with PG were affected by polyunsaturated fat.

One property of LDL that is thought to be important in atherosclerosis progression and LDL–PG interaction is the apoE content (6). The apoE content of plasma LDL is highly correlated with coronary artery atherosclerosis in nonhuman primates (7). ApoE also contains two sites that bind to the glycosaminoglycan, heparin (14). In a previous study, the apoE content of LDL and the density distribution of LDL subfractions appeared to be related to the decreased interaction of FO LDL with PG compared to Lard LDL (6). However, the results from the present study show that Poly LDL was similar to Lard LDL and Mono LDL in chemical composition (Table 4), density distribution (Table 2) and apoE content (Table 3). The data taken together suggest that FO decreases PG binding *in vitro* by decreasing the apoE content and amount of the d = 1.015–1.025 g/mL subfraction, whereas Poly appears to affect LDL–PG interactions by other mechanisms.

A possible explanation for differences in LDL–PG interaction may relate to LDL particle charge. LDL sialic acid content varies among LDL subfractions (28) and among individuals (29,30). The sialic acid content of LDL also influences PG interaction (29). We are aware of no data that suggest that the type of dietary fat modifies the sialic acid content of LDL, and we found no difference in LDL sialic acid content among the four diet groups in this study (Table 6). Therefore, the observed differences in LDL–PG interaction among diet groups cannot be explained by differences in sialic acid content.

In the present study, the type of dietary fat had little effect on the chemical composition, density distribution, size and apoE content of plasma LDL, except in the FO group. FO LDL were smaller (Table 1), more dense (Table 2) and contained less apoE (Table 3) and less CE (Table 4) compared to LDL from the other three diet groups. The decrease in size and CE content of FO LDL likely resulted from decreased CE input into plasma LDL by the two CE-generating enzymes, lecithin:cholesterol acyltransferase

(LCAT) and acyl-CoA:cholesterol acyltransferase (ACAT). Previous studies have shown that phospholipids containing n-3 fatty acids in the *sn*-2 position are poor substrates for LCAT regardless of whether the phospholipid is in a recombinant substrate particle or a monolayer film (31,32). In addition, livers from nonhuman primates fed FO diets secrete less cholesterol than those from animals fed lard diets (33), and hepatic microsomal ACAT activity is reduced compared to animals fed lard and safflower oil diets (23). HDL phospholipids purified from the plasma of cynomolgus monkeys fed the FO diet and made into recombinant substrate particles are less reactive with LCAT than plasma HDL phospholipids isolated from the Lard, Poly or Mono diet groups (Thornburg, J.T., Parks, J.S., and Rudel, L.L., manuscript in preparation). Taken together, these data suggest that FO LDL are smaller, more dense and contain fewer CE molecules per particle because n-3 fatty acids are poor substrates for CE synthesis by LCAT and ACAT.

The decreased apoE content of FO LDL and LDL subfractions with respect to the other diet groups may be related to a decreased association of apoE with the LDL particle surface. Relative to Lard LDL, FO LDL are enriched in sphingomyelin and relatively poor in phosphatidylcholine, and the phosphatidylcholine species are enriched in n-3 fatty acids. These FO-induced changes in the LDL particle surface may limit apoE binding. In support of this hypothesis, we have found that apoA-I binds more poorly to phosphatidylcholine monolayers containing n-3 fatty acids vs. oleic acid in the *sn*-2 position (34). In addition, Ibdah *et al.* (35) have demonstrated that the increased sphingomyelin content of LDL vs. HDL may prevent apoA-I binding to LDL. If apoE binding to lipoprotein surfaces is influenced by FO in a manner similar to that for apoA-I, then the FO-induced changes in LDL phospholipids may reduce apoE binding to LDL. Studies are currently underway to test this possibility.

The LDL apoE/B molar ratio was greater in this study compared to our previous study (6). Although the explanation for this difference is unknown, it likely relates to the amount of dietary cholesterol consumed by the animals. Diets containing 0.26 mg cholesterol/kcal were used in the previous study compared to 0.4 mg cholesterol/kcal in the present one. This may have contributed to the nearly twofold greater concentration of apoE in whole plasma in the present study compared to that observed previously. In addition, different animals were used for the two studies. In spite of the difference in apoE enrichment of LDL between the studies, the effect of dietary FO on LDL size, composition, density distribution and PG interaction was similar compared to the Lard group in both studies.

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Fatty Acid Composition of Lung, Macrophage and Surfactant Phospholipids After Short-Term Enteral Feeding with n-3 Lipids

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Utilization of enteral feeding modalities may prove clinically relevant for rapid modulation of lung phospholipid polyunsaturated fatty acids (PUFA) that serve as substrates for the formation of vasoactive dienoic eicosanoids. We compared the effects of short-term enteral feeding with formulations enriched with either fish (n-3) or corn (n-6) oil PUFA on the fatty acid composition of rat lung, alveolar macrophage and surfactant phospholipids. The diets were infused continuously for 72 h through a surgically placed gastroduodenal feeding catheter by a syringe pump. The n-3 PUFA derived from the fish oil enriched diet were readily incorporated into the phospholipid membranes of the alveolar macrophages, lung tissue and pulmonary surfactant. The relative percentages of the n-3 PUFA were significantly higher and individual and total n-6 PUFA significantly lower in the macrophage, lung and surfactant phospholipids from the n-3-supplemented rats in comparison with those present in the rats infused enterally with the n-6 diet or untreated, chow-fed rats (baseline). In contrast, there was a significant increase in linoleic acid (18:2n-6) without modification of arachidonic acid (20:4n-6) in the alveolar macrophages, lung tissue and surfactant from rats enterally receiving the n-6 diet relative to levels measured in the rats at baseline. The results suggest that short-term continuous delivery of n-3-enriched enteral preparations can foster rapid modification of membrane phospholipid PUFA composition of lung tissue, alveolar macrophages and lung surfactant. Utilization of similar infusion modalities to deliver n-3-enriched enteral formulations may prove beneficial to critically ill or postoperative patients with persistent lung inflammation secondary to uncontrolled formation of vasoactive eicosanoids derived from arachidonic acid.
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Arachidonic acid (20:4n-6, AA), an n-6 fatty acid derived from the essential dietary fatty acid linoleic acid (18:2n-6; LA), serves as the primary precursor of vasoactive eicosanoids formed *via* cyclooxygenase and 5′-lipoxygenase within immune cell (e.g., macrophage) membrane phospholipids. The resulting leukotrienes and dienoic series of prostanoids are potent mediators of lung injury and inflammation promoting platelet aggregation, arteriolar vasoconstriction, capillary permeability and neutrophil (PMN) chemotaxis (1,2). Given the extensive capillary vasculature and large population of marginating PMN that are normally adherent to lung endothelium (3), the lung is particularly vulnerable to tissue damage by PMN and capillary occlusion by activated platelets after trauma or sepsis. Persistent lung inflammation, with or without infection, further compromises pulmonary endothelial integrity, impairs gas exchange and predisposes the development of adult respiratory distress syndrome (ARDS) (4). In theory, then, critically ill patients at risk of developing ARDS may derive some benefit from treatment modalities that reduce lung generation of, or exposure to, AA-derived eicosanoids.

Clinical and experimental studies conducted during the past decade have revealed that the structural and functional properties of host immune cells can be modified by nutritional supplementation with marine oil (n-3) polyunsaturated fatty acids (PUFA), e.g., eicosapentaenoic acid (20:5n-3; EPA) (5,6). Studies from this laboratory have shown that displacement of n-6 PUFA, i.e., 20:4n-6 and 18:2n-6, from plasma phospholipids by n-3 PUFA in endotoxin-challenged guinea pigs was associated with reduced pulmonary leukosequestration, improved lung morphology and increased survival (7,8). Others have reported that P_aO_2 and blood pressure were significantly higher and that plasma thromboxane A_2 was lower during an infusion of live *Escherichia coli* in pigs prefed an n-3-enriched diet relative to corresponding values in corn oil-fed animals (9). These studies indicate that nutritional modulation of cell membrane PUFA with marine oil n-3 fatty acids appears to confer an advantage with respect to maintenance of pulmonary function during septic complications.

In the clinical setting, critically ill or surgical patients often require enteral nutrition support secondary to anorexia and/or a postoperative incapacity (e.g., upper gastrointestinal surgery) to consume adequate amounts of nutrients. Unfortunately, the majority of commercially available enteral nutrient preparations are enriched with 18:2n-6, thus predisposing incorporation of 20:4n-6 within macrophage membrane phospholipids. Therefore, utilization of novel enteral formulations containing marine oil PUFA to modulate the phospholipid PUFA composition and function of immune cells is now being explored. We, and others, have demonstrated that continuous enteral delivery of n-3-enriched formulations promotes significant displacement of 18:2n-6 and 20:4n-6 from liver Kupffer and endothelial cells and plasma phospholipids in animals (10) as well as from erythrocyte phospholipids in humans (11,12) within 4–7 d, irrespective of concurrent endotoxemic or septic complications. Recent studies of the effects of infusion time have shown that the modification of macrophage phospholipid PUFA achieved after three days of contin-

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; ARDS, adult respiratory distress syndrome; CSP, crude surfactant pellet; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DPPC, dipalmitoyl phosphatidylcholine; EPA, eicosapentaenoic acid; LA, linoleic acid; LPS, lipopolysaccharide; MUFA, monounsaturated fatty acids; PBS, phosphate buffered saline; PMN, polymorphonuclear neutrophil; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

uous enteral delivery of diets containing fish and borage oil PUFA was not enhanced by feeding for three additional days (13). Other studies from this laboratory have confirmed that systemic levels of dienoic prostanoids elicited after endotoxin (lipopolysaccharide; LPS) exposure were significantly lower in rats enterally fed with a fish oil-enriched formulation than in LPS-challenged rats infused with a safflower oil-based diet (14).

The extent to which short-term infusions of enteral formulations enriched with fish oil modify lung tissue, alveolar macrophages and surfactant phospholipid PUFA has not been addressed. The ability to induce dietary modifications of surfactant PUFA may also prove important, given that the relative percentage of 20:4n-6 in surfactant phospholipids increases in patients with ARDS (15,16). The objectives of the present study were to determine changes in the individual n-3 and n-6 PUFA within the phospholipid fractions of these lung components from rats fed enterally for 72 h with fish or corn oil-enriched formulations containing moderate (27%) amounts of lipid.

MATERIALS AND METHODS

Animal surgery. The study design was approved by the Deaconess Animal Care and Use Committee. The methods and diets were identical to those described previously (10). Briefly, male (200–250 g) Sprague-Dawley rats (Harlan, Altamont, NY) were allowed standard chow pellets (Prolab RMH 3000; Agway Inc., Cornell, NY) *ad libitum* for five days prior to study entry. One group of rats (n = 8) was then sacrificed to provide baseline levels of lung tissue, alveolar macrophage and surfactant phospholipid PUFA. The remaining rats (n = 16) underwent aseptic surgical placement of an enteral feeding catheter. After anesthetization with diethyl ether (08:30 h of day 0), a 0.030″ × 0.065″ medical silastic catheter was advanced into the duodenum through an ostomy in the fundus of the stomach and anchored to the stomach wall with a purse string suture. The proximal end of the catheter was tunneled subcutaneously, exteriorized at the midscapular region and attached to a flow-through swivel to permit freedom of movement. Each rat was housed individually in a wire-bottomed cage during enteral feeding and allowed water *ad libitum* for the duration of the study.

Enteral feeding. After recovery from surgery, the rats received either a fish oil- or a corn oil-enriched enteral formulation based upon preoperative randomization (n = 8/group). The enteral diets consisted of a complete basal formulation (Vital HN; Ross Laboratories, Columbus, OH) with which an isocaloric quantity of either fish oil concentrate (Omegacaps Ltd., St. Louis, MO) or corn oil (Best Foods, Union, NJ) was blended. The fatty acids from the fish oil concentrate were primarily in the form of ethyl esters. The absorption of fish n-3 PUFA given as ethyl esters has been shown to be similar to that of fish n-3 PUFA given as triglycerides (17). The corn oil was supplemented with α-tocopherol to equal the total vitamin E activity present in the fish oil concentrate (1 U/g oil). Each enteral diet provided 1.4 g N/kg/d and 250 kcal/kg/d. Lipids accounted for 27% of the total dietary energy in the enteral formulations. The calories derived from lipid in the chow pellets constituted 14.5% of total dietary energy. The relevant fatty acids of the enteral formulations and chow pellet (baseline) triglycerides were determined by gas chromatography and expressed as relative percentage of the total fatty acids present (Table 1). The enteral diets were infused continuously by a programmable syringe pump (Model 42; Harvard Apparatus, Natick, MA) at a constant rate for 72 h beginning at 13:00 h of day 0.

Tissue procurement. Each rat was anesthetized with an intraperitoneal injection of pentobarbital (80 mg/kg B.W.) at 13:00 of day 3. The thoracic cavity was exposed and the lung vasculature flushed with 5 mL saline *via* the superior *vena cava*. A 14-gauge angiocatheter was inserted through a tracheostomy, and the lungs were lavaged eight times with 5 mL of cold phosphate buffered saline (PBS); gentle chest massage facilitated lavage recovery (>90%). The cumulative lavage fluid was kept on ice during this procedure. Following lavage, lung tissue samples (free of bronchial tissue) were excised, minced, washed with PBS and stored under N_2 at −20°C until analysis. Frozen lung tissue was homogenized in saline immediately before lipid extraction, as described in the next paragraph.

The lavage fluid was spun at 400 × *g* for 10 min at 4°C. The cell-free supernatant was then respun at 40,000 × *g* at 4°C for 60 min to obtain a crude surfactant pellet (CSP) (18). The supernatant was removed, and the CSP was resuspended in 200 µL of saline containing 5 mM $CaCl_2$ for total phospholipid fatty acid analysis. The cell pellet containing alveolar macrophages after the first centrifugation at 400 × *g* was resuspended in PBS and centrifuged at 110 × *g* for 10 min at 4°C. The washed cell pellet was then resuspended in PBS, layered on Ficoll Paque (Pharmacia, Piscataway, NJ) and centrifuged at 500 × *g* at 22°C for 35 min to isolate the alveolar macrophages. The macrophage layer was removed, washed with PBS, centrifuged at 110 × *g* for 10 min at 4°C and resuspended in PBS. Purity and viability (trypan blue exclusion) were >95% under light microscopy. Alveolar macrophages (5–7 × 10^6) were stored in PBS at −20°C for total phospholipid fatty acid analysis.

TABLE 1

Fatty Acid Composition of Baseline and Enteral Diets[a]

Fatty acid	Baseline	Corn oil-enriched	Fish oil-enriched
<14:0	2.1	4.3	6.7
16:0	20.2	9.3	2.5
18:0	8.7	1.8	2.0
18:1n-9	29.7	24.4	6.8
18:2n-6	34.6	54.4	12.9
18:3n-3	0.3	0.7	0.6
20:5n-3	1.2	0.2	21.7
22:5n-3	0.1	0.1	4.2
22:6n-3	1.1	0.1	16.2

[a]The baseline diet was chow pellets. The corn oil- and fish oil-enriched diets were enteral formulations. Relative percent of total fatty acids as determined by gas chromatography.

Phospholipid fatty acid analysis. Sixteen identifiable fatty acids (12–24 carbons in length) in the phospholipid fraction of lung tissue homogenate, CSP and alveolar macrophages were determined by methods described previously (10). Diheptadecanoyl phosphatidylcholine was used as an internal standard and was added prior to lipid extraction. After lipid extraction in chloroform/methanol (2:1, vol/vol), the phospholipid fraction was isolated on silica gel H (Analtech, Newark, DE) thin-layer plates using a mobile phase of petroleum ether/diethyl ether/glacial acetic acid (80:20:1, by vol) and identified relative to the migration of a phospholipid standard using dichlorofluorescein spray. The phospholipid band was isolated and the fatty acids were transmethylated with 14% BF_3 in methanol. In a few instances, smaller bands of surfactant phospholipids from identically treated rats were combined to improve yield. The fatty acid methyl esters were separated and quantified on a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph equipped with a 50-m fused silica capillary column containing SP-2330 as the stationary phase with a 0.20 mm film thickness (Supelco, Bellefonte, PA). The relative mole percentages of individual fatty acids were determined using Chem Station software (Hewlett-Packard) based upon the relative responses of an external standard of pure fatty acid methyl esters (Nu-Chek-Prep, Elysian, MN).

Statistics. Computer-based statistical programs were used (BMDP Software, Inc., Los Angeles, CA). Statistical tests included analysis of variance (ANOVA) by diet. Levene's test provided a test of equal variability among diet groups. If the Levene's test was significant, the Brown–Forsythe test was used to allow for unequal group variances at the expense of a loss of degrees of freedom. If the overall ANOVA or Brown–Forsythe test was significant ($P < 0.05$), *post hoc* comparisons of means were determined using Bonferroni comparison tests.

RESULTS

The enteral formulations used were well tolerated by all the rats. There were no signs of physical stress or diarrhea during the enteral feeding. Spleen weights and overall morphologic appearance of the major organs were similar to the baseline group given chow pellets *ad libitum*. There were no significant differences between the two groups of enterally fed rats with respect to either initial (overall mean ± SD = 230 ± 7 g) or final (231 ± 8 g) body weights.

Lung phospholipid PUFA. The relative mole percentage of the principal fatty acids present in the phospholipids of the lung homogenate are listed in Table 2. Despite the fact that the enteral diets contained relatively lower levels of 16:0 than the chow pellets (Table 1), a trend toward an increased relative percentage of 16:0 and total saturated fatty acids (SFA) in the enterally-fed rat lungs relative to that in the baseline group was evident. This rise in the relative percentage of 16:0 was slightly offset by a reduction of lung phospholipid 18:0 after enteral feeding in comparison to the relative percentage of 18:0 in the baseline group of rats. The relative percentage of total phospholipid PUFA in the lungs was similar among the three diet groups. Total monounsaturated fatty acids (MUFA), the bulk of which is rep-

TABLE 2

Phospholipid Fatty Acids of Lung Tissue[a]

Fatty acid	Baseline	Corn oil diet	Fish oil diet	ANOVA	Bonferroni P value[b]		
					B vs. C	B vs. F	C vs. F
14:0	1.6 ± 0.1	2.2 ± 0.5	2.8 ± 1.0	0.01	NS	0.01	NS
16:0	38.1 ± 2.7	41.6 ± 2.9	43.7 ± 4.7	NS[c]			
16:1n-7	1.8 ± 0.1	1.6 ± 0.3	1.9 ± 0.3	NS			
18:0	14.7 ± 0.7	13.0 ± 1.3	12.2 ± 1.5	NS			
18:1n-9	11.3 ± 0.6	10.0 ± 1.0	9.2 ± 1.1	0.001	NS	0.001	NS
18:2n-6	7.9 ± 0.5	10.3 ± 0.8	6.5 ± 0.5	0.001	0.001	0.001	0.001
20:3n-6	0.9 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	0.001	0.05	0.001	0.01
20:4n-6	13.4 ± 0.9	12.4 ± 1.0	8.6 ± 1.5	0.001	NS	0.001	0.001
20:5n-3	1.3 ± 0.3	0.1 ± 0.2	5.0 ± 1.0	0.001	0.05	0.001	0.001
22:5n-3	2.7 ± 0.7	1.4 ± 0.5	3.0 ± 0.8	0.001	0.01	NS	0.001
22:6n-3	2.2 ± 0.4	1.4 ± 0.2	3.2 ± 0.5	0.001	0.05	0.001	0.001
SFA	56.0 ± 2.3	58.7 ± 2.3	60.4 ± 4.2	NS			
MUFA	14.6 ± 0.6	14.2 ± 2.1	12.5 ± 1.3	0.01	NS	0.05	NS
Total n-3 PUFA	6.7 ± 1.2	3.0 ± 0.8	11.4 ± 1.4	0.001	0.001	0.001	0.001
Total n-6 PUFA	22.7 ± 1.3	24.1 ± 1.8	15.7 ± 2.1	0.001	NS	0.001	0.001
Total PUFA	29.4 ± 1.8	27.1 ± 1.9	27.1 ± 3.1	NS			
20:5n-3/20:4n-6	0.1 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.001	NS	0.001	0.001
n-3/n-6	0.3 ± 0.1	0.1 ± 0.0	0.7 ± 0.1	0.001	0.001	0.001	0.001

[a]All values are expressed as mean mole % ± SD; n = 8/group. ANOVA, analysis of variance; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]Bonferroni comparisons listed are between baseline (B), corn oil-enriched (C) and fish oil-enriched (F) diets.
[c]NS, not significant.

resented by 18:1n-9, differed significantly between the fish and chow groups only (12.5 vs. 14.2%, respectively).

Provision of the fish oil PUFA enriched diet for 72 h induced significant changes in the individual and total n-3 and n-6 PUFA in the lung phospholipids relative to those present in the baseline (chow) group or those infused with the corn oil PUFA diet (Table 2). Both 18:2n-6 and 20:4n-6, as well as total n-6 PUFA, were significantly lower in the lung phospholipids of the rats given the fish oil PUFA diet than in those measured in the lungs from either the corn oil group or baseline group. The mean relative percentage of 18:2n-6 in lungs from the fish group was 6.5% as compared with 10.3 and 7.9% in lungs from the corn and baseline groups, respectively. The relative percentage of 20:4n-6 in the lung phospholipids of the fish oil group was 8.6%, in comparison to 12.4% in the corn oil group ($P < 0.001$) or 13.4% in the baseline group ($P < 0.001$). In conjunction with these reductions, the relative percentage of individual n-3 PUFA, the 20:5n-3/20:4n-6 ratio, the n-3/n-6 ratio, and the sum of n-3 PUFA were significantly higher ($P < 0.001$) in the fish group relative to those of either the corn or baseline group. The mean 20:5n-3 in the lung phospholipids of the rats given the fish oil-enriched diet enterally was 5.0% as compared to 1.3% at baseline and 0.1% in the corn oil-enriched groups, respectively. Lung phospholipid docosapentaenoic (DPA; 22:5n-3) acid (3 vs. 1.4%) and docosahexaenoic (DHA; 22:6n-3) acid (3.2 vs. 1.4%) were also higher in the group given the fish oil diet relative to the rats given the corn-oil-enriched diet.

It was also observed that the baseline rats had a significantly higher relative percentage of lung phospholipid 20:5n-3 and total n-3 PUFA than the corn group; this difference was presumably due to the presence of fish meal in the chow pellets used. These results also suggest that continuous enteral feeding for 72 h with the corn-oil-enriched diet, which was devoid of n-3 PUFA, promoted rapid loss of phospholipid 20:5n-3 and 22:6n-3 originally present after the baseline feeding period.

Alveolar macrophage phospholipid PUFA. The effects of enteral infusion of the fish- or corn oil-enriched formulations on the fatty acid composition of alveolar macrophage phospholipids (Table 3) paralleled those observed in the lung tissue. There were no differences among the three diet groups with respect to the relative percentage of individual SFA. However, the relative percentage of total SFA in alveolar macrophages from the enterally infused rats tended to be higher than the total SFA at baseline (65 vs. 61%). Concurrently, the total PUFA in alveolar macrophage phospholipids from both groups of enterally fed rats were lower than those measured at baseline. Total MUFA were similar among the three dietary groups. As observed in the lung tissue analyses (Table 2), the alveolar macrophages from the fish group had significantly lower relative percentage of 18:2n-6 and 20:4n-6 and greater relative percentage of 22:6n-3 than the macrophages from either the baseline rats or those infused with the corn oil-enriched formulation. The rats receiving the fish oil-enriched diet also had significantly higher 20:5n-3/20:4n-6 and n-3/n-6 ratios and total n-3 PUFA in the alveolar macrophage phospholipids than those in the rats at baseline or after enteral feeding with the corn oil-enriched diet. Rats given the corn oil-enriched diet for 72 h had significantly higher ($P < 0.001$) relative percentage of 18:2n-6 than baseline rats (8.9 vs 6.8%, respectively). As observed in the lung

TABLE 3

Phospholipid Fatty Acids of Alveolar Macrophages[a]

Fatty acid	Baseline	Corn oil diet	Fish oil diet	ANOVA	Bonferroni P value[b]		
					B vs. C	B vs. F	C vs. F
14:0	2.1 ± 0.9	3.4 ± 1.3	3.3 ± 1.4	NS[c]			
16:0	43.2 ± 5.4	44.5 ± 5.4	46.7 ± 4.6	NS			
16:1n-7	2.0 ± 0.7	1.6 ± 1.2	1.1 ± 0.6	NS			
18:0	15.0 ± 1.6	16.1 ± 4.6	13.2 ± 1.2	NS			
18:1n-9	7.7 ± 1.0	6.4 ± 1.9	4.8 ± 1.4	0.001	NS	0.01	NS
18:2n-6	6.8 ± 0.7	8.9 ± 1.4	3.5 ± 0.6	0.001	0.001	0.001	0.001
20:4n-6	13.8 ± 1.7	12.4 ± 2.8	8.1 ± 0.6	0.001	NS	0.001	0.01
20:5n-3	1.6 ± 1.9	0.0 ± 0.0	2.9 ± 1.1	0.01	0.05	NS	0.001
22:5n-3	1.8 ± 1.4	0.5 ± 0.5	2.9 ± 0.5	0.001	0.01	NS	0.001
22:6n-3	3.9 ± 2.2	1.4 ± 0.7	6.4 ± 1.4	0.001	0.01	0.01	0.001
SFA	61.2 ± 4.5	65.6 ± 3.2	65.4 ± 1.7	NS			
MUFA	10.8 ± 1.4	11.0 ± 3.4	11.0 ± 2.3	NS			
Total n-3 PUFA	7.3 ± 5.1	1.9 ± 1.0	12.3 ± 2.0	0.001	0.01	0.05	0.001
Total n-6 PUFA	20.6 ± 2.2	21.3 ± 2.2	11.6 ± 0.4	0.001	NS	0.001	0.001
Total PUFA	28.0 ± 4.0	23.2 ± 2.5	23.6 ± 1.9	0.01	0.01	0.05	NS
20:5n-3/20:4n-6	0.0 ± 0.0	0.2 ± 0.2	0.6 ± 0.4	0.01	NS	0.001	0.05
n-3/n-6	0.4 ± 0.3	0.1 ± 0.1	1.0 ± 0.2	0.001	0.05	0.001	0.001

[a]All values are expressed as mean mole % ± SD; n = 8/group. For abbreviations, see Table 2.
[b]Bonferroni comparisons listed are between baseline (B), corn oil-enriched (C), and fish oil-enriched (F) diets.
[c]NS, not significant.

tissue phospholipids, concomitant levels of alveolar macrophage phospholipid 20:4n-6 and total n-6 PUFA in the rats infused with the corn oil-enriched diet remained similar to those seen in the baseline group (Table 3).

Surfactant phospholipid fatty acids. In general, compositional changes in the fatty acid profiles of the (SP) phospholipids (Table 4) after enteral feeding followed the trends observed for both the lung tissue (Table 2) and the alveolar macrophages (Table 3). The relative percentage of 16:0, the primary fatty acid associated with phosphatidylcholine, the main surfactant phospholipid, was comparable among the three diet groups. Although the baseline diet contained a higher relative percentage of 18:0 than either enteral diet (Table 1), surfactant phospholipids from the enterally fed rats, particularly those receiving the fish oil diet, had significantly higher levels of this SFA. The increase in surfactant SFA in the enteral fish group relative to the baseline rats was matched by a corresponding decrease in total MUFA, similar to the changes observed in lung tissue (Table 2). The relative percentage of both 18:2n-6 and 20:4n-6 were significantly lower in the surfactant phospholipids from the rats receiving the fish oil enteral formulation in comparison to surfactant from either the rats at baseline or the rats fed enterally with the corn oil formulation. The relative percentage of 20:5n-3, 22:6n-3 and total n-3 PUFA changed inversely to the relative percentage of individual and total n-6 PUFA. Significant displacement of n-6 PUFA from the phospholipids by n-3 PUFA was exemplified by the increased ratios of both 20:5n-3/20:4n-6 and n-3/n-6. However, the total PUFA in surfactant phospholipids remained similar among the diet groups.

DISCUSSION

Novel enteral formulations containing n-3 PUFA derived from marine and plant oils are currently being evaluated for potential utilization in critically ill or postoperative patients (11–13). Recent clinical studies have revealed that surgical (11,19) or burn (20) patients provided with fish oil-enriched formulations experienced reductions in the number of infections per patient and/or lengths of stay relative to patients that received standard formulations enriched with LA. The efficacy of long-chain n-3 PUFA to attenuate a post-traumatic inflammatory response is partly due to their capacity to displace n-6 PUFA from immune cell membrane phospholipids. In addition, EPA (20:5n-3), the principal fish oil n-3 PUFA, attenuates the formation of proinflammatory dienoic eicosanoids by competing with 20:4n-6 for cyclooxygenase binding sites (21). The eicosanoids derived from 20:5n-3 have reduced inflammatory properties relative to those derived from 20:4n-6. EPA also retards the conversion of 18:2n-6 to 20:4n-6 by inhibiting the rate-limiting enzyme, Δ6 desaturase. Other studies have demonstrated that cytokine release was lower from stimulated mononuclear cells that were enriched with n-3 PUFA than in cells containing higher relative percentage n-6 PUFA (5). However, the efficacy of n-3-enriched enteral diets for attenuating lung injury or inflammation in the clinical setting remains unresolved. Moreover, the extent of dietary n-3 PUFA uptake by lung tissue and alveolar components has not been assessed in patients nutritionally supported with n-3-enriched enteral preparations.

TABLE 4

Phospholipid Fatty Acids of Surfactant[a]

Fatty acid	Baseline	Corn oil diet	Fish oil diet	ANOVA	Bonferroni *P* value[b]		
					B vs. C	B vs. F	C vs. F
14:0	4.8 ± 0.8	4.0 ± 0.7	4.1 ± 0.2	NS[c]			
16:0	73.7 ± 3.0	76.6 ± 3.1	77.2 ± 3.8	NS			
16:1n-7	3.6 ± 0.4	1.7 ± 0.4	1.2 ± 0.8	0.001	0.001	0.001	NS
18:0	2.7 ± 0.3	4.0 ± 0.8	5.3 ± 1.0	0.001	0.05	0.001	0.05
18:1n-9	3.4 ± 0.7	2.5 ± 0.9	1.6 ± 1.3	0.05	NS	0.05	NS
18:2n-6	5.9 ± 1.7	6.6 ± 1.4	2.4 ± 0.9	0.001	NS	0.01	0.001
20:4n-6	3.0 ± 0.6	2.5 ± 0.5	1.1 ± 0.3	0.001	NS	0.001	0.01
20:5n-3	0.4 ± 0.1	0.0 ± 0.0	1.7 ± 0.6	0.01	NS	0.001	0.001
22:5n-3	0.4 ± 0.1	0.0 ± 0.1	0.3 ± 0.3	NS			
22:6n-3	1.4 ± 0.2	0.5 ± 0.4	3.4 ± 0.1	0.001	0.01	0.001	0.001
SFA	81.3 ± 3.3	85.2 ± 2.4	87.2 ± 3.2	0.025	NS	0.05	NS
MUFA	7.5 ± 0.8	5.2 ± 0.7	3.9 ± 1.1	0.001	0.01	0.001	NS
Total n-3 PUFA	2.2 ± 0.3	0.6 ± 0.4	5.4 ± 0.8	0.001	0.01	0.001	0.001
Total n-6 PUFA	9.0 ± 2.3	9.1 ± 1.9	3.5 ± 1.3	0.001	NS	0.01	0.001
Total PUFA	11.2 ± 2.5	9.6 ± 1.8	8.9 ± 2.1	NS			
20:5n-3/20:4n-6	0.1 ± 0.0	0.0 ± 0.0	1.5 ± 0.1	0.001	0.05	0.001	0.001
n-3/n-6	0.3 ± 0.1	0.1 ± 0.1	1.7 ± 0.4	0.001	NS	0.001	0.001

[a]All values are expressed as mean mole % ± SD; n = 8/group. For abbreviations, see Table 2.
[b]Bonferroni comparisons listed are between baseline (B), corn oil-enriched (C) and fish oil-enriched (F) diets.
[c]NS, not significant.

The present study was conducted to characterize the incorporation of n-3 and n-6 PUFA by rat lung, alveolar macrophage and surfactant phospholipids after short-term enteral infusion of diets enriched with fish or corn oil. The results revealed that continuous enteral feeding with a fish oil-enriched formulation fostered significant modulation of the PUFA composition of rat lung parenchymal tissue (Table 2), macrophage (Table 3) and surfactant (Table 4) phospholipids within 72 h. The generalized effects were that n-6 PUFA (i.e., 18:2n-6 and 20:4n-6), which are potential precursors of the vasoactive dienoic eicosanoids and leukotrienes, were displaced by longer chain n-3 PUFA (i.e., 20:5n-3, 22:5n-3 and 22:6n-3). The rapidity of these changes paralleled those previously observed in the plasma pool, as well as liver sinusoidal cells of rats after short-term enteral feeding with identical formulations (10). These findings may have clinical relevance given that equations formulated to predict tissue incorporation of dietary n-3 PUFA at specific intake ratios of n-3/n-6 PUFA are similar for rats and humans (22). Moreover, we have also observed that the incorporation of n-3 fatty acids into rat liver or lung macrophages during continuous enteral feeding is not impaired during concurrent endotoxemia (10,13). Evidence from clinical trials supports these findings. Other investigators (12) have shown that the relative percentage of EPA and n-3/n-6 ratios in both plasma and erythrocytes from critically ill patients were increased significantly above baseline within seven days after continuous enteral administration of a fish oil-enriched formulation.

In contrast to the rats infused with the n-3-enriched diet in the present study, rats infused with the corn oil-enriched formulation exhibited significant increases of 18:2n-6 with concomitant reductions of 20:5n-3 and total n-3 PUFA in lung (Table 2) and alveolar macrophage (Table 3) phospholipids relative to initial levels measured in the baseline rats given chow pellets. The importance of these results is underscored in that the majority of moderate- to high-fat enteral formulations currently used in the clinical setting have percentages of 18:2n-6 similar to that present in the corn oil-enriched formulation. The relative percentage of 20:4n-6 in both lung and alveolar macrophage phospholipids after enteral treatment with the corn oil diet remained unchanged from levels measured at baseline. The results from this study support the concept that significant displacement of 20:4n-6 from phospholipids by dietary PUFA is negligible unless: (i) the exogenous dietary PUFA have a carbon length or degree of unsaturation similar to 20:4n-6, and (ii) the relative n-3/n-6 ratio of the dietary PUFA is favorably weighted to enhance exchange with endogenous PUFA (23,24). With regard to the first conditional requirement, we had previously observed that liver sinusoidal cell and plasma phospholipid 18:2n-6, but not 20:4n-6, could be displaced by 18:1n-9 in rats infused with an enteral formulation enriched with olive oil (10).

The data from rats given the diet enriched with fish oil PUFA suggest that alveolar macrophages may be more capable of metabolizing 20:5n-3 (EPA) and/or 22:5n-3 (DPA) to 22:6n-3 (DHA) than lung tissue. Using the mean values listed in Tables 2 and 3, rough estimates of the EPA/DHA ratios for lung tissue and alveolar macrophages are 1.6 and 0.4, respectively. Further examination of the data reveals that the corresponding ratios of EPA/DPA are 1.7 and 1.0, respectively, and the ratios of DPA/DHA are 0.9 and 0.4, respectively, for lung and alveolar macrophage phospholipids. These ratios suggest that the alveolar macrophage appears to possess relatively higher elongase (i.e., for conversion of EPA to DPA) as well as $\Delta 4$ desaturase activity (i.e., for conversion of DPA to DHA) than the lung. However, such estimates based upon relative percentage of substrate do not take into account any potential differences that may exist between lung tissue and alveolar macrophages with regard to their individual capacities to incorporate DPA and/or DHA present in the diet (Table 1). With this *caveat* in mind, alveolar macrophages appear to be similar to peritoneal macrophages with respect to elongase activity (25). However, the $\Delta 4$ desaturase activity of peritoneal macrophages has been reported to be low (26). Further studies are required to determine any actual differences in enzyme activities between these two types of macrophages.

An interesting finding in the present study was that although alveolar macrophages and surfactant are not in direct physical contact with the plasma pool, displacement of their respective phospholipid n-6 PUFA by dietary n-3 PUFA during the 72 h infusion period was remarkable. Whether these alveolar components acquired n-3 PUFA transported directly from the plasma or through an intermediary cell source, e.g., type I or II pneumocytes, has not been resolved. In support of the latter concept, other investigators have recently reported that incorporation of dietary n-3 PUFA into type II pneumocytes, which synthesize surfactant, readily occurred *in vivo* in rats given chow enriched with fish oil (27). Moreover, an earlier study documented the transfer of radiolabeled 20:4n-6 from alveolar type II cells to alveolar macrophages *in vitro* (28). Alternatively, some portion of the alveolar macrophage phospholipid 22:5n-3 and/or 22:6n-3 may have been derived from elongation and/or desaturation of 20:5n-3 (as discussed earlier) within the macrophage or by the type II cell. Presumably, these latter cells also served as the direct source of the n-3 PUFA that were present in the surfactant phospholipids.

Although the possibility exists that the alveolar macrophages were newly recruited from the vascular pool of monocytes enriched with n-3 PUFA, an earlier study of bone marrow transplant patients indicated that replacement of resident alveolar macrophages by those derived from the transplanted marrow had occurred over several months (29). Alternatively, a source of n-3 PUFA for alveolar macrophages may, in part, arise from the surfactant phospholipid pool (Table 4). Phospholipids account for 80% of the composition of pulmonary surfactant. Moreover, these macrophages actively phagocytose surfactant as a regulatory mechanism for surfactant clearance (30,31). However, surfactant turnover would have to proceed at a rate high enough to en-

able uptake of significant amounts of n-3 PUFA by the macrophages. In this regard, earlier investigations indicated that >90% of radiolabelled surfactant phosphatidylcholine was cleared from the alveolar space within 24 h in adult rabbit lungs *in vivo* (32). Unfortunately, the metabolic complexity of and potential interactions among the lung's alveolar cells and components obviate identification of the immediate source(s) of n-3 PUFA present in the alveolar macrophages at this time.

We were unable to measure surfactant surface tension, precluding detection of potential dietary effects arising from n-3 PUFA on this functional parameter. Dipalmitoyl phosphatidylcholine (DPPC) is the primary surfactant phospholipid involved in the reduction of alveolar surface tension (31,33). In this regard, the relative percentage of palmitic acid (16:0) present in the surfactant total phospholipids was similar among the treatment groups (Table 4). However, it is not known whether changes in surfactant DPPC concentration occurred. A recent study of rats fed a fish oil-enriched diet for four weeks revealed that the amount of DPPC present in the surfactant was similar to that present in rats given a corn oil-enriched diet (27); however, DPPC associated with lung tissue from the fish oil group was increased relative to that in the corn oil group in that study. Another recent study (34) indicated that pretreatment of pigs with an n-3-enriched diet did not alter the minimum surface tension of pulmonary surfactant after endotoxin challenge relative to that measured in pigs fed an n-6-enriched diet. Given these findings, it appears unlikely that modification of the unsaturated fatty acid profile of surfactant phospholipids by dietary treatment with n-3 PUFA would alter or impair the surface tension properties of lung surfactant.

In summary, significant displacement of both LA and AA from rat lung, alveolar macrophage and surfactant phospholipids was achieved within 72 h by continuous delivery of an enteral formulation enriched with fish oil PUFA. The capacity of enteral feeding modalities to rapidly modulate the PUFA composition of these lung components may facilitate timely attenuation of eicosanoid-driven inflammatory processes in critically ill or postoperative patients at risk of developing ARDS.

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Effect of Dietary Linseed Oil on Tumoricidal Activity and Eicosanoid Production in Murine Macrophages

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Diets that contain high levels of n-3 fatty acids from fish oil have been shown to significantly effect macrophage cytolytic capacity, tumor necrosis factor alpha production and eicosanoid production. The present study was undertaken to determine whether n-3 fatty acids from vegetable origin [linseed oil (LIN)] would have the same effects on murine macrophage tumoricidal capacity and eicosanoid production as would fish oil. Mice were fed for three weeks diets that contained 10% (wt/wt) of either LIN, which is high in linolenic acid (18:3n-3), menhaden fish oil (MFO), which is high in eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, or safflower oil (SAF), which is high in linoleic acid (18:2n-6). *In vivo*- or *in vitro*-activated macrophages were assessed for select functions. As expected, macrophages from mice fed LIN and MFO produced significantly lower levels of both prostaglandins and leukotriene C_4 when compared with macrophages from mice fed SAF. In addition, LIN and MFO macrophages were able to synthesize leuko-triene C_5, which could not be produced by macrophages from mice fed SAF. The effects of LIN, however, were not as pronounced as those of MFO. With respect to specific functions, macrophages from mice fed LIN did not have altered cytolytic capacity when compared with macrophages from mice fed SAF and activated *in vitro* with either lipopolysaccharide (LPS) alone for 24 h or with LPS plus interferon gamma (IFNγ) for 5 h. Diet did not significantly alter tumoricidal capacity of macrophages activated completely *in vivo* either. Specific binding of macrophages to tumor targets, nitric oxide production and the production of tumor necrosis factor alpha were found to be unaltered by LIN when compared with SAF. The results are not consistent with a general n-3 effect, as LIN could not effect the functions comparable to MFO. The results also suggest that a change in eicosanoid production may not be sufficient to modulate tumoricidal activity in macrophages.

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Dietary n-3 fatty acids have attracted much attention of late as it has been shown that they can modulate the immune system and, in particular, macrophage function (1–5). Fish oil, which contains high levels of the n-3 fatty acids, eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, is a major source of n-3 fatty acids. Another source of n-3 fatty acids is linseed oil (LIN), which contains high levels of the n-3 fatty acid, α-linolenic acid (18:3n-3). Although there are numerous reports on the effects of fish oil on immune and, specifically, macrophage function, only very few have focused on the effects of LIN. One report (6) described the effect of LIN on macrophage phagocytosis of yeast and carbon particles and on macrophage arginase activity; however, no differences in phagocytic capacity were observed among the diets tested (LIN and corn oil), although arginase activity was decreased with LIN feeding. The authors (6) suggested the decreased arginase activity was due to a decrease in prostaglandin E (PGE) production caused by the LIN diet. The effects of dietary LIN on macrophage tumoricidal capacity have not been studied. We, and others, have reported, however, on the effects of menhaden fish oil (MFO) on macrophage cytolytic capacity (7), peroxide production and Iα expression (3,8), as well as on tumor necrosis factor alpha (TNFα) production (5,9–11). These studies showed that n-3 fatty acids from MFO could have contrasting effects on macrophage function. For example, although MFO was shown to decrease macrophage responsiveness to interferon gamma (IFNγ), and thus, cytolytic capacity (7), it was shown to increase macrophage capacity for TNFα production (4,5,10). In the present study, we show the effects of dietary LIN, which is also high in n-3 fatty acids, on macrophage tumoricidal activity and eicosanoid production. We used two populations of macrophages that have a high capacity for cytolytic activity. One population, thioglycollate-elicited macrophages, have been well-characterized but must be stimulated *in vitro* before they can kill tumor cells. The other population of macrophages are elicited with maleic anhydride divinyl ether (MVE-2) and lipopolysaccharide (LPS) and do not require activation *in vitro* as they are fully activated for tumor cell kill *in vivo* (12).

MATERIALS AND METHODS

Diets and animals. Female C57BL/6NCr mice, five weeks of age, were obtained from Charles River (Kingston, NY). Animals were housed in autoclaved cages in a laminar flow hood and given sterilized water to minimize "spontaneous" activation of macrophages (13). Mice were fed standard chow upon arrival, but were switched to the experimental diets after 48 h. Mice were fed the semipurified diets for at least three weeks before experiments were performed. The diets tested were adequate in all nutrients and varied only in the type of oil fed, i.e., either LIN (Seymour Organic Foods, Seymour, IL), safflower oil (SAF; California Oils, Richmond, CA), or MFO, which made up 10% of the diet by weight. The basic compositions of the semipurified diets have been described previously in detail (7); the diets contained casein, 20%; DL-methionine, 0.3%; corn starch, 15%; sucrose, 44%; cellulose, 5%; AIN-76 mineral mix, 3.5%; AIN-76 vitamin mix, 2%;

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Abbreviations: HBSS, Hank's balanced salt solution; IFNγ, interferon gamma; MEM, minimum essential medium; PEC, peritoneal exudate cells; PGE, prostaglandin E; LIN, linseed oil; LPS, lipopolysaccharide; LT, leukotriene; MFO, menhaden fish oil; MVE-2, maleic anhydride divinyl ether; SAF, safflower oil; SDS, sodium dodecylsulfate; TNFα, tumor necrosis factor alpha.

choline chloride, 0.2%; and the dietary oil, 10%. The major fatty acids of the SAF diet were (in wt%): 16:0, 7.0%; 18:0, 2.5%; 18:1n-9, 10.7%; 18:2n-6, 78.2%; and others, 1.6%. The LIN diet contained 16:0, 6.5%; 18:0, 3.9%; 18:1n-9, 22.1%; 18:2n-6, 17.0%; 18:3n-3, 47%; and others, 3.5%. The MFO diet contained 14:0, 7.7%; 16:0, 16.0%; 18:0, 2.8%; 18:1n-9, 10.5%; 18:2n-6, 1.2%; 18:3n-6, 0.6%; 20:4n-6, 0.6%; 20:5n-3, 16.9%; 22:5n-3, 2.5%; 22:6n-3, 12.0%; and others, 29.2%. The powdered diets were mixed and stored at –20°C under N_2. Animals were allowed free access to both food and water. Neither body weights nor food intake differed significantly ($P > 0.05$) between the three dietary groups (data not shown).

Macrophages. Thioglycollate-elicited macrophages were obtained 3–5 d after peritoneal injection of 2.0 mL sterile fluid thioglycollate medium (prepared to manufacturer's specifications; Difco, Detroit, MI). For fully activated macrophages, mice were injected with 500 μg MVE-2 (Hercules, Inc., Wilmington, DE) plus 10 ng/mL LPS 5 d prior to use. Peritoneal exudate cells (PEC) were harvested from no less than three mice for each experiment with Hank's balanced salt solution (HBSS; BioWhittaker, Walkersville, MD) and ones from similar diet groups were pooled, centrifuged at 500 × *g* for 5 min, and resuspended in Eagle's minimum essential medium (MEM; BioWhittaker) containing 5% heat-inactivated calf serum (Hyclone, Logan, UT). Macrophage concentrations were adjusted after differential staining. There were no dietary fat effects on the percentage of PEC that were macrophages, regardless of the elicitation method or PEC yield (data not shown). Following 90 min of adherence in Eagle's MEM plus 5% serum, nonadherent cells were removed by rinsing with HBSS, and macrophage monolayers were further incubated in serum-free Eagle's MEM with or without activating agents, as described next. Macrophages made up more than 98% of the final adherent cell population as judged by phagocytosis and morphology. There were no differences in the adherence efficiency between the dietary groups (data not shown). The endotoxin level of the serum was <0.06 ng/mL, as determined by the supplier; all other reagents were negative for endotoxin as determined by the *Limulus* amebocyte lysate assay (BioWhittaker) which was sensitive to <0.1 ng/mL. All glassware was heat-sterilized at 180°C for 4 h to degrade residual endotoxin.

Analysis of eicosanoid production. For the measurement of leukotrienes (LT) produced, thioglycollate-elicited macrophages were isolated from PEC as described previously by adherence to 100-mm tissue culture plates. Cells were rinsed, then incubated in serum-free MEM (alpha modification; BioWhittaker) supplemented with 20 mM [*N*-2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid], 2 mM L-glutamine, 5 μg/mL gentamicin and 5 mM reduced glutathione for 2 h (14). After purification, macrophages were stimulated with 5 μM calcium ionophore A23187 for 40 min, and supernatants were immediately frozen at –75°C. Leukotrienes were analyzed essentially as previously described (15). Briefly, frozen supernatants were thawed and extracted using BakerBond reversed-phase C18 extraction columns (J.T. Baker, Phillipsburg, NJ). Extracted samples were dried under nitrogen and resuspended in a small aliquot of methanol/water (1:1, vol/vol). Samples were separated on a Waters 600E high-performance liquid chromatography using a Resolve C18 reversed-phase column (Waters, Milford, MA) and a mobile phase of methanol/5 mM ammonium acetate (3:1, vol/vol) run at 0.55 mL/min. Peaks were compared with authentic standards and verified by spectral analysis using an on-line photodiode array detector (Waters). Leukotrienes were quantitated by peak comparison with that of a prostaglandin B_2 standard. Levels of PGE_2 produced were measured in macrophage supernatants using an enzyme immunoasay kit (Cayman Chemical Co., Ann Arbor, MI). Protein was measured using the Biorad protein assay (Biorad, Hercules, CA).

Cytolysis assay and nitric oxide production. Thioglycollate-elicited macrophages from mice fed the experimental diets were plated in 96-well dishes and were either activated 24 h with LPS (*Escherichia coli* serotype 0111:B4, phenol extract, 0-1000 ng/mL; Sigma, St. Louis, MO), or 5 h with LPS, IFNγ (0–25 U/mL; Gibco, Gaithersburg, MD), or LPS + IFNγ in serum-free Eagle's MEM. Fully activated macrophages were plated in the same manner, but because they were completely activated *in vivo* for cytolysis, they received no additional treatment. Monolayers of *in vitro*- or *in vivo*-activated macrophages were overlaid with ^{51}Cr-labeled P815 cells as described (7). Briefly, P815 targets in midlog phase of growth (5×10^5/mL) were incubated for 60 min in 0.5 mL Eagle's MEM + 5% CS with 100 μCi/mL $Na_2{}^{51}CrO_4$ (NEN, Boston MA) with frequent gentle agitation. The cells were washed with HBSS, then resuspended to 10^5 cells/mL in Eagle's MEM + 5% serum. Aliquots were added to macrophage monolayers to give an effector/target ratio of 10:1, and the cultures were incubated for 18 h. To assess cytolysis, cell-free supernatant was removed from each well and placed into a glass tube for counting in a gamma spectrophotometer. Spontaneous release of label was determined from wells containing tumor cells alone, and total incorporated radioactivity was released by lysis with 0.5% sodium dodecylsulfate (SDS). Nitric oxide produced was measured in the supernatants of the cytolysis experiments using Greiss reagent (16). Briefly, macrophage supernatants were reacted with equal parts of 1% sulfanilamide (Sigma) and 0.1% naphthylethylenediamine (Sigma). The spectral change was determined on a microplate spectrophotometer at 550 nm, and the sample concentrations (nmoles nitrite/10^6 cells) calculated using a standard curve created with known concentrations of pure nitrite. Cytolysis and nitric oxide studies were done in triplicate, and each experiment was repeated three times. The results presented come from one representative experiment. Other experiments showed similar trends.

Selective binding. Thioglycollate-elicited macrophage monolayers in 96-well plates were treated with LPS, IFNγ or LPS + IFNγ as in the cytolysis assay described previously. Radiolabeled P815 cells were then added for various periods of time from 15 to 90 min, after which nonadherent P815 cells were removed by vigorous rinsing. Monolayers with adherent P815 cells were then lysed with 0.5% SDS and an aliquot counted in the gamma counter.

TNFα bioassay. Thioglycollate-elicited macrophages from diet mice were plated in 96-well dishes and treated

with LPS, as just described. After the activation period, supernatants of monolayers were tested for TNFα content by using a bioassay with the TNFα-sensitive cell line L929, as described (2,17). For the bioassay, sample supernatants or rTNFα standard were added in triplicate to 96-well plates containing 2–3 × 10^4 adherent L929 cells. Twofold serial dilutions were made, and the wells were then overlaid with Eagle's MEM containing 10% serum and 5 μg/mL actinomycin D (Sigma). The plates were incubated for 20 h, rinsed and stained with 0.2% crystal violet in 2% EtOH, dried, treated with 0.5% SDS, and quantitated at 570 nm. TNFα activity was calculated as the reciprocal dilution at 50% lysis of L929 cells. Sensitivity of the assay for TNFα was tested by treating some samples containing TNFα with a polyclonal rabbit anti-mouse TNFα antibody for neutralizing (Genzyme) which, at a dilution of 1:100, completely inhibited cytolysis of L929 targets. All bioassays were repeated at least three times.

Statistical analyses. Statistical analysis was performed using a two-tailed Student's *t*-test. Differences were judged as statistically significant when the *P* value was less than 0.05. The number of mice used for each experiment depended upon the number of cells required, the eliciting agent and the peritoneal macrophage yield.

RESULTS

Eicosanoid production. Eicosanoids were measured: (i) to allow evaluation of the extent to which dietary fat could modulate macrophage fatty acid composition; (ii) to compare the modulation seen with that observed previously with dietary fish oil; and (iii) to help in elucidating the mechanism by which dietary fat affects macrophage functions, many of which are modulated by eicosanoids. To address the changes caused by LIN feeding, we tested LT and PGE production by thioglycollate-elicited macrophages before and after stimulation with calcium ionophore A23187. Feeding mice LIN significantly decreased ($P < 0.05$) A23187-stimulated thioglycollate-elicited macrophage leukotriene C_4 (LTC_4) production when compared to macrophages from mice fed SAF (Table 1). Moreover, although there was no production of leukotriene C_5 (LTC_5) in macrophages from mice fed SAF, LIN macrophages produced relatively small amounts of LTC_5 that were significantly ($P > 0.05$) greater than those produced by SAF macrophages (Table 1). This alteration of LT production by LIN, however, was not as great as that seen with MFO (Table 1 and Ref. 15). Linseed oil feeding also altered PGE production by thioglycollate-elicited macrophages when compared with macro-phages from mice fed SAF. Upon stimulation with A23187, LIN macrophages produced 3–4 times less PGE when compared with A23187-stimulated SAF macrophages (Table 2). Macrophages from mice fed MFO showed further decreased PGE production compared with both LIN and SAF mice. We and others have previously shown that MFO feeding significantly decreased PGE production by macrophages (7,10). Basal levels of PGE were not significantly ($P > 0.05$) altered by dietary fat.

Cytolysis. Other n-3 fatty acids have been shown to alter macrophage cytolytic capacity (7). It has not been resolved, however, whether the effects of dietary MFO are directly due to alterations in eicosanoid metabolism, or to some other mechanism. Because LIN can also alter macrophage eicosanoid production (results above), we first tested the effects of LIN on *in vitro* activation of similar thioglycollate-elicited macrophages for tumoricidal activity against P815 mastocytoma cells. LIN feeding did not alter cytolysis after short-term *in vitro* activation with LPS and IFNγ (Fig. 1) or long-term *in vitro* activation with LPS only (data not shown). MFO feeding, however, significantly ($P < 0.05$) altered cytolysis when macrophages were incubated with relatively low levels of IFNγ and LPS (10 ng/mL). At higher levels of IFNγ, the cytolytic capacity of macrophages from mice fed MFO was similar to that of LIN and SAF macrophages. The results of cytolysis with MFO macrophages are consistent with our previously published data (7). Because fatty acids may be secreted from the macrophages and because culture conditions may alter cellular lipid levels during activation, macrophages were also activated *in vivo* with MVE-2/LPS. Tumoricidal macrophages obtained directly from mice fed LIN, SAF or MFO were cytolytic but did not differ in their capacity to kill tumor cells (data not shown).

TABLE 1

Effect of Dietary Fat on Leukotriene Production in Thioglycollate-Elicited Murine Macrophages[a]

Dietary fat	LTC_4	LTC_5
	(ng · mg protein^{-1} · h^{-1})	
Linseed oil	38 ± 7[b]	9 ± 2
Safflower oil	82 ± 11	ND[c]
Menhaden fish oil	40 ± 3[b,d]	24 ± 6[d]

[a]Measured leukotriene levels represent means ± SEM, and are based on triplicate samples from separate mice. Leukotriene production was stimulated *in vitro* with A23187 (5 μM).
[b]Significantly ($P < 0.05$) decreased as compared to levels from safflower oil-fed group.
[c]ND, not detected.
[d]Published data (Ref. 15) are presented here for comparison only.

TABLE 2

Effect of Dietary Fat on Prostaglandin E (PGE) Production in Thioglycollate-Elicited Murine Macrophages[a]

Dietary fat	Unstimulated	Stimulated
	(pg · mg protein^{-1} · h^{-1})	
Linseed oil	350 ± 80	1000 ± 160[b,c]
Safflower oil	290 ± 30	3550 ± 860[b]
Menhaden fish oil[c]	300 ± 30	520 ± 70[b–d]

[a]The enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) used is for PGE_2 and cross-reacts minimally with PGE_1 and 100% with PGE_3. Macrophages were stimulated with calcium ionophore A23187 for 40 min and the supernatants tested directly using the kit.
[b]Significantly ($P < 0.05$) greater when compared with unstimulated cells.
[c]Significantly ($P < 0.05$) lower when compared with stimulated cells from the safflower oil-fed group.
[d]Significantly ($P < 0.05$) lower when compared with stimulated cells from the linseed oil-fed group.

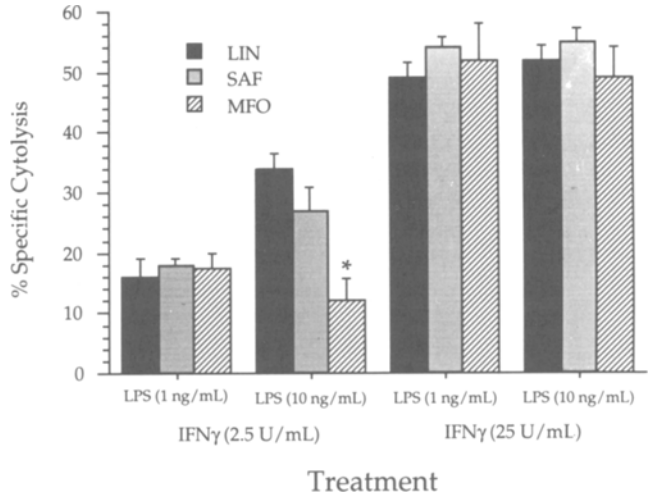

FIG. 1. Effect of dietary fat on *in vitro* activation of thioglycollate-elicited macrophages for specific tumor cell cytolysis. Macrophages from mice fed the different diets were activated with interferon gamma (IFNγ) and lipopolysaccharide (LPS) for 5 h in serum-free medium. *Significantly ($P < 0.05$) decreased compared to the other diets at that treatment. LIN, linseed oil; SAF, safflower oil; MFO, menhaden fish oil.

Nitric oxide production and selective binding. It is possible that while functional macrophage cytolysis was not affected by LIN feeding, some of the capacities required for cytolysis, such as tumor cell binding or nitric oxide production, may be differentially affected such that the sum of their alteration may add up to no effect. Our results, however, show this not to be the case as dietary fat did not alter the production of nitric oxide, a cytolytic mediator elaborated by macrophages (Fig. 2). To bring about cytolysis, activated macrophages must first specifically bind to target cells (18). When radiolabeled P815 cells were added to macrophages activated with 1 ng/mL LPS and 25 U/mL

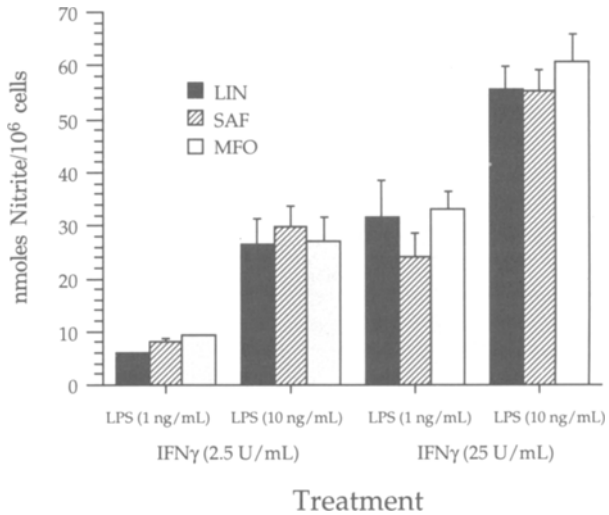

FIG. 2. Effect of dietary fat on *in vitro* activation of thioglycollate-elicited macrophages for nitric oxide production. Macrophages from diet mice were treated with IFNγ and LPS for 5 h, then overlaid with the tumor targets for 18 h. Nitric oxide was measured from the supernatants after that time period. No significant ($P > 0.05$) differences among the diets were observed. Abbreviations as in Figure 1.

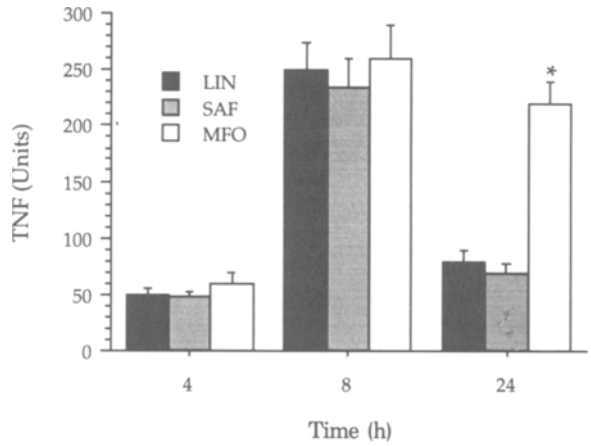

FIG. 3. Effect of dietary fat on the production of tumor necrosis factor alpha (TNFα) by LPS-stimulated (10 ng/mL) thioglycollate-elicited macrophages. TNFα bioactivity was calculated as the reciprocal dilution at 50% lysis of L929 cells. *Significantly ($P < 0.05$) increased as compared to the other diets at that time point. Abbreviations as in Figure 1.

IFNγ, no significant differences ($P > 0.05$) were observed between the dietary groups in the percentage of tumor targets that were specifically bound (LIN = 16 ± 3; SAF = 21 ± 4). No differences were observed at other levels and combinations of LPS and IFNγ, such as 10 and 100 ng/mL LPS, and 0.25 and 2.5 U/mL IFNγ (data not shown).

TNFα production. Dietary MFO has been shown to modulate TNFα production, which may be due to alterations in eicosanoid production (5,11). When we tested the effects of LIN-feeding, which alters eicosanoid production, on bioactive TNFα levels in LPS-stimulated (10 ng/mL) thioglycollate-elicited macrophages, we found that there were no significant ($P > 0.05$) differences as compared to the SAF group (Fig. 3). MFO, in contrast, was able to alter macrophage TNFα production at 24 h, which is consistent with our previous data (5).

DISCUSSION

In the present study, we have shown that feeding 10% LIN significantly altered eicosanoid production, but was unable to alter specific macrophage cytolytic capacity against P815 mastocytoma cells or TNFα production. These results suggest that the mechanism of the effects on macrophage function due to dietary fat may be independent of altered eicosanoid synthesis, as LIN does alter eicosanoid synthesis, but does not alter cytolysis. It is well established that feeding animals fish oil can alter the production of eicosanoids by macrophages and other cells. In general, fish oil feeding suppresses PGE_2 production and four-series leukotriene production in macrophages while stimulating the synthesis of new five-series leukotrienes. Few studies prior to this report have sought to evaluate the effects of other n-3 fatty acid sources on eicosanoid production (19–21). In one study (19), a diet containing 18:3n-3 was shown to decrease peritoneal macrophage production of leukotriene B_4 (LTB_4) and LTC_4, as well as PGE_2. Recently it was also shown that a diet containing 18:3n-3 can induce *in vivo* peritoneal macrophages stimu-

lated with zymosan to synthesize leukotriene E_5 (20,21). In our study, we showed that thioglycollate-elicited macrophages from mice fed LIN, which contains relatively high levels of 18:3n-3 (47%), were able to produce LTC_5 after stimulation with calcium ionophore A23187. Although a limited number of studies have begun to define the roles of LTB_4 and PGE_2 in the immune system, the effects of LTC_4 and LTC_5 have not been clarified. The mechanism by which dietary fat alters eicosanoids and its relationship to macrophage function has yet to be determined, although it may be due to altered tissue levels of arachidonate, the precursor for eicosanoids.

Three previous studies (22–24) have reported on the effects of dietary LIN on the production of TNFα by macrophages. In one, LIN feeding was shown to increase TNFα production in rat resident macrophages as compared to macrophages from animals fed corn oil (22). However, in that same study, no differences were observed among the diets with respect to TNFα production by complete Freund's Adjuvant-elicited macrophages. In another study (23), resident and casein-elicited, but not thioglycollate-elicited, peritoneal macrophages from mice fed diets containing high levels of 18:2n-3 produced greater amounts of TNFα compared with macrophages from mice fed diets containing high levels of 18:2n-6. Neither did we see an enhancement of TNFα production by thioglycollate-elicited macrophages with LIN feeding. It has been shown that PGE can significantly down-regulate production of TNFα by macrophages, whereas inhibition of PGE synthesis has the opposite effect (5,10,25). Therefore, a possible mechanism for the effect of fish oil on TNFα production by macrophages may be related to an alteration in eicosanoid synthesis. LIN feeding, however, does not have the same capacity to affect TNFα production in thioglycollate-elicited macrophages, perhaps because it does not decrease PGE production to the same extent as MFO.

Besides modifying the production of TNFα in macrophages (4,5,10), dietary MFO can significantly alter activation of thioglycollate-elicited macrophages for cytolysis of tumor targets (7). These effects may have been due to the levels of n-3 fatty acids in the fish oil. One possible mechanism for the effects of dietary fat on tumoricidal activity may be an alteration of eicosanoid synthesis, as others have shown that PGE is responsible for down-regulating resident macrophage tumoricidal capacity (26–28). In the present study, however, we did not observe an alteration in cytolysis with LIN feeding after either *in vitro* activation with IFNγ and LPS or *in vivo* activation with MVE-2. Perhaps LIN did not alter eicosanoid production to the extent required for down-regulation.

In the present study we have attempted to clarify whether n-3 fatty acids have a general effect on macrophage function. Because LIN contains significant levels of 18:3n-3, and 18:3n-3 can be converted to 20:5n-3, we expected to observe effects similar to those previously reported for fish oil. With respect to eicosanoid production, LIN did have an effect similar to that of MFO. However, with respect to macrophage tumoricidal capacity, LIN feeding followed the pattern of the SAF (n-6) effects. Thus, the relationship between the effects of n-3 fatty acids and macrophage function may be specific for the type of n-3 fatty acid, or may be responsive to the balance between n-3 and n-6 fatty acids.

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COMMUNICATION

Inhibition of Cytolytic T Lymphocyte Activity by Oxysterols

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The objective of this study was to investigate the effects of oxysterols (OS), namely 5α-hydroxy-6-ketocholestanol, 6-ketocholestanol and 25-hydroxycholesterol, on specific cell-mediated cytotoxicity by C57BL/6 spleen cells against P815-X2 (a DBA/2 mastocytoma) target cells. Cytolytic T lymphocytes (CTL) were generated by intraperitoneally injecting C57BL/6 mice with P815-X2 tumor cells 10 d prior to the cytotoxicity experiments. Preincubation of CTL with 10^{-5} M 5α-hydroxy-6-ketocholestanol and 6-ketocholestanol for 45 min in lipoprotein-depleted medium resulted in an inhibition of cytolytic activity (73 and 43%, respectively) as measured by 4-h ^{51}Cr release. At a concentration of 5×10^{-6} M, 5α-hydroxy-6-ketocholestanol inhibited CTL activity by 65%, whereas 6-ketocholestanol did not elicit any inhibition. By contrast, 25-hydroxycholesterol did not inhibit CTL at either concentration, although it is known to be a potent inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway. When CTL were preincubated with OS in lipoprotein-replete medium, there was no inhibition of CTL activity at the respective concentrations. The results suggest that the inhibition of CTL activity upon short-term incubation with OS is not due to the inhibition of cholesterol synthesis, but may be due to the insertion of OS into the plasma membrane to replace cholesterol and alteration of membrane physical properties.
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Oxysterols (OS) are cholesterol oxidation products which may be produced under a variety of conditions that are associated with oxidant stress (1–7). Increased generation of OS has been reported to occur in the cell membrane under prooxidant conditions (6). Cholesterol oxidation products have been found in skin exposed to ultraviolet light (3,4), which is known to have immunosuppressive effects. Grever *et al.* (8) observed that after exposure to highly reactive oxygen compounds human lymphocytes were significantly impaired in both antibody-dependent and nonantibody-mediated cytotoxicity against a human lymphoblast target cell. As OS have been shown to inhibit cellular immunity (9–11), it is possible that the oxidant induced inhibition of cell-mediated toxicity observed by Grever *et al.* (8) may be due to generation of OS in the cell membrane. OS taken up by diet may also be immunosuppressive, as was shown by Heiniger *et al.* (12), who observed that a diet containing 0.25% 7-ketocholesterol had a suppressive effect on the secondary immune response in C57BL/6 mice.

We have previously reported the inhibitory effect of OS on natural killer (NK) cell activity (11). In the present report, we describe the effects of short term *in vitro* incubation with three OS, namely 25-hydroxycholesterol, 6-ketocholestanol and 5α-hydroxy-6-ketocholestanol, on tumor cell killing by cytolytic T lymphocytes (CTL) specifically generated *in vivo* against the target allogeneic tumor cells. We chose two OS that are oxidized at the sterol nucleus and one OS that is oxidized at the alkyl side chain, in order to determine if the location of the oxygenated group influences the effects of OS on CTL activity. We also chose to study these OS because they are among the oxidation products of cholesterol (1) and because their effects on other systems have been studied (11,13).

MATERIALS AND METHODS

Animals. Normal 4–6-week-old male C57BL/6 (H-2^b) mice were obtained from Harlon (Frederick, MD). The animals were age-matched (6–10 weeks old) at the onset of each experiment.

OS. 6-Ketocholestanol (3β-hydroxy-5α-cholestan-6-one) was obtained from Sigma (St. Louis, MO) and 5α-hydroxy-6-ketocholestanol (3β,5-dihydroxy-5α-cholestan-6-one) and 25-hydroxycholesterol (cholest-5-ene-3β,25-diol) were obtained from Steraloids (Wilton, NH). The OS were added to lymphocyte suspensions as ethanolic stock solutions with 100 times the desired final concentration.

Target cells. P815-X2 mastocytoma cells (DBA/2 origin, H-2^d) were maintained in tissue culture flasks containing RPMI-1640 medium with Hepes (MA Bioproducts, Walkersville, MD), 5% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 0.1 mg/mL gentamycin and 5 mM L-glutamine in a 5% CO_2 humidified incubator. Cultures were passaged twice weekly and periodically tested for mycoplasma contamination.

Preparation of lymphocytes. Ten days after an intraperitoneal inoculation of 20×10^6 viable P815-X2 cells, spleens were removed from C57BL/6 mice immediately after cervical dislocation. Single cell suspensions of spleen cells were made in ice-cold RPMI-1640 with gentle teasing of cells out of the spleens by using a scalpel blade. After enrichment of the lymphocyte fraction using an Isolymph (Gallard-Schlesinger Chemical, Carle Place, NY) gradient, the cells were washed, and 2×10^6 cells in 4 mL of RPMI-1640 were placed in tubes for incubation.

Incubation with OS. One mL of lipoprotein-depleted serum (LPDS) or normal human AB serum (ABS) was added to 4 mL of lymphocyte suspension, resulting in 20% lipoprotein-depleted medium (LPDM) or lipoprotein replete medium. LPDS was prepared as previously de-

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Abbreviations: ABS, human blood group AB serum; CTL, cytolytic T lymphocyte; H-2, mouse major histocompatibility complex; HMG–CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPDM, lipoprotein-depleted medium; LPDS, lipoprotein-depleted serum; NK, natural killer; OS, oxysterol.

scribed (11) by ultracentrifugational flotation of lipoproteins of a density <1.25 g/mL. The residual bottom fraction was dialyzed extensively against phosphate buffered saline, heated to 56°C for 45 min, and sterilized by membrane filtration prior to use. Fifty μL of an ethanolic solution of OS was added, resulting in a final OS concentration ranging from 2.5×10^{-6} M to 10^{-5} M. Because OS were added in ethanol, all incubations contained a final concentration of 1% (vol/vol) ethanol. Incubations were carried out at 37°C for 45 min with gentle rocking in a water bath. Viability of lymphocytes was assessed by trypan blue dye exclusion both before and after incubation.

^{51}Cr release assay. P815-X2 target cells (10^6 cells) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 90 min at 37°C and were washed three times. The desired concentration of lymphocytes (100 μL) and 100 μL of ^{51}Cr-labeled target cells (5,000) were added into triplicate wells of a 96-well, U-bottomed microtiter plate (Corning, Corning, NY) to obtain the desired effector/target cell ratios. The plates were centrifuged at $300 \times g$ for 2 min and then incubated for 4 h at 37°C under a 5% CO_2 atmosphere in a humidified incubator. The plates were then centrifuged at $500 \times g$ for 10 min, and 100 μL of supernatant was transferred to tubes for radioactivity counting. The percentage of specific ^{51}Cr release was calculated by using the formula:

$$\frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm maximal release} - \text{cpm spontaneous release}} \times 100 \quad [1]$$

in which maximal release refers to the cpm obtained after adding Triton X-100 (0.5%) to the target cells just before centrifugation of the plates, and spontaneous release refers to the cpm released by target cells in the absence of effectors. All experimental and control values were the means of three replicates.

Percent inhibition of CTL activity by OS was calculated by using the formula:

$$\frac{\%\ \text{specific}\ ^{51}\text{Cr release with control medium} - \%\ \text{specific}\ ^{51}\text{Cr release with OS}}{\%\ \text{specific}\ ^{51}\text{Cr release with control medium}} \times 100 \quad [2]$$

Control medium contained 1% ethanol and 20% LPDM with no OS added. Percent inhibition values shown on Table 1 are the results of four experiments expressed as mean % inhibition ± standard error.

RESULTS

Table 1 shows the inhibition of cytolytic T cell activity by the OS used in this study. At a concentration of 2.5×10^{-6} M in the preincubation medium, none of the OS resulted in an inhibition of CTL activity. At a concentration of 5×10^{-6} M, 5α-hydroxy-6-ketocholestanol resulted in a 65% inhibition of CTL activity. When the concentration of OS in the incubation medium was increased to 10^{-5} M, 6-ketocholestanol resulted in a 43% inhibition of CTL activity, and the inhibition with 5α-hydroxy-6-ketocholestanol increased to 73%. 25-Hydroxycholesterol resulted in little or no inhibition of CTL activity at any of the above concentrations used in these experiments. The viability of lymphocytes after incubation with OS at these concentrations was more than 90%, excluding the possibility of OS toxicity.

TABLE 1

Concentration-Dependent Inhibition of Specific Cytolytic T Lymphocyte (CTL) Activity by 5α-Hydroxy-6-ketocholestanol and 6-Ketocholestanol

	% of inhibition[a] of CTL by OS at different concentrations		
Oxysterols (OS)	2.5×10^{-6} M	5.0×10^{-6} M	1.0×10^{-5} M
5α-Hydroxy-6-ketocholestanol	8 ± 6[b]	65 ± 20	73 ± 15
6-Ketocholestanol	10 ± 6	8 ± 4	43 ± 20
25-Hydroxycholesterol	4 ± 3	4 ± 3	0

[a]% Inhibition = [(% specific ^{51}Cr release with control medium − % specific ^{51}Cr release with OS)/(% specific ^{51}Cr release with control medium)] × 100.
[b]Results of four experiments are expressed as percentage of inhibition (mean ± SE). Effector cell to target cell ratio is 50:1.

TABLE 2

Presence of Lipoproteins (ABS) in the Incubation Medium Prevents Inhibition of CTL Activity by 5α-Hydroxy-6-ketocholestanol (5×10^{-6} M)[a]

Percentage specific ^{51}Cr release (mean ± SE)[b]		
20% LPDS + ethanol	20% LPDS + OS	20% ABS + OS
57 ± 2	29 ± 14	51 ± 4

[a]Abbreviations as in Table 1. LPDS, lipoprotein-depleted serum; ABS, human blood group AB serum.
[b]Results of four experiments are expressed as mean percentage of specific ^{51}Cr release ± SE. Effector cell to target cell ratio is 50:1.

The inhibitory effect of 5α-hydroxy-6-ketocholestanol on CTL activity was prevented by the presence of lipoproteins in the incubation medium, which was achieved by using normal human ABS instead of lipoprotein-depleted serum in the incubation medium (Table 2). At 5×10^{-6} M 5α-hydroxy-6-ketocholestanol in the preincubation medium, % specific ^{51}Cr release was 29 ± 14% in the presence of 20% LPDM and 51 ± 4% (mean ± standard error) in the presence of 20% ABS. Control incubation with 20% LPDM and 1% ethanol in the absence of 5α-hydroxy-6-ketocholestanol resulted in a % specific ^{51}Cr release of 57 ± 2%. Results are the means ± standard errors of four experiments under each condition at an effector/target cell ratio of 50:1.

DISCUSSION

In the present study we have examined the effects of OS on specific CTL activity. The nature of inhibition of specific CTL activity by OS was similar to the previously reported (11) inhibition of NK cell activity by these compounds, i.e., OS oxidized at the C-5 and C-6 positions of the sterol nucleus inhibited CTL activity, whereas 25-hydroxycholesterol being oxidized at C-25 of the alkyl side chain had little or no inhibitory effect (Table 1). However,

CTL appear to be less sensitive to the inhibitory effects of OS as compared to NK cells, which were inhibited more profoundly under similar experimental conditions (11). When the insertion of OS into the plasma membrane of CTL was reduced due to the presence of lipoproteins in the incubation medium, there was little inhibition of CTL activity (Table 2). This finding supports the hypothesis that OS inhibit CTL lytic function by inserting themselves into the plasma membrane and altering its physical properties. This phenomenon of lipoprotein competition with plasma membrane for OS binding was also observed in the OS-induced inhibition of NK cell activity (11).

OS may influence cellular functions either by altering the physical properties of the membrane (e.g., fluidity) due to their presence in the lipid bilayer as cholesterol analogues, or by inhibiting 3-hydroxy-3-methylglutaryl CoA (HMG–CoA) reductase, the key regulatory enzyme in the cholesterol biosynthetic pathway (13–15), or by other yet unknown means. Heiniger *et al.* (9) have shown that preincubation of CTL generated in secondary C57BL/6 anti-DBA/2 mixed lymphocyte cultures with an OS inhibitor of cellular cholesterol biosynthesis (25-hydroxycholesterol) for 24 h strongly depressed cytolytic activity. In other studies involving similar long-term incubations with OS, Humphries and McConnell (16) have observed inhibition of plaque-forming cell response by 25-hydroxycholesterol, and Spangrude *et al.* (10) observed inhibition of differentiation and function of tumor specific cytotoxic T lymphocytes by 25-hydroxycholesterol, 20α-hydroxycholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol. Although 20α-hydroxycholesterol and 25-hydroxycholesterol caused a parallel depression in lymphocyte proliferation, CTL activity and endogenous sterol biosynthesis, 7β-hydroxycholesterol and 7α-hydroxycholesterol abrogated CTL activity without depressing proliferative responses and endogenous sterol biosynthesis (10). We have previously found that oxidation of cholesterol at C-5, C-6 and C-7 of the sterol nucleus results in OS which are potent inhibitors of NK cell-mediated cytotoxicity (11).

The mechanism of inhibition of CTL activity by OS is unlikely to be due to inhibition of cholesterol synthesis in these experiments, because: (i) CTL activity was not inhibited by 25-hydroxycholesterol (Table 1), which is a potent inhibitor of HMG–CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis; and (ii) the duration of exposure to OS was too short (45 min) to attribute the inhibition of CTL to decreased cholesterol synthesis. Previously observed morphologic and fluidity changes in red cell membranes (6,13,15,17) and the functional membrane changes in lymphocytes (18) and granulocytes (19) induced by OS suggest that the inhibition of CTL may be due to alterations in the physical properties of CTL plasma membrane caused by OS. Figure 1 depicts the OS used in this study. The presence of OS in a cell membrane would be expected to affect the packing of phospholipid molecules. Model membrane (20,21) and red blood cell membrane (6,15,22) studies have shown that OS influence membrane fluidity and lipid packing. The inhibition of CTL activity by OS oxidized at the C-5 and C-6 positions, but not by OS oxidized at C-25 position, appears to be consistent with the hypothesis that inhibition of CTL by OS

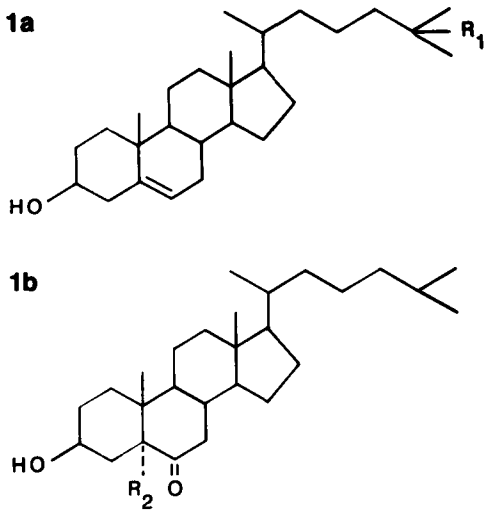

FIG. 1. Schematic representation of the sterols used. (1a) Cholesterol: R_1 = H; 25-hydroxycholesterol: R_1 = OH; (1b) 6-ketocholestanol: R_2 = H; 5α-hydroxy-6-ketocholestanol: R_2 = OH.

is due to its effects on fluidity and/or packing of the plasma membrane, which would be expected to be affected more profoundly by OS oxidized at the sterol nucleus (i.e., C-5, C-6), as compared to OS oxidized at the alkyl side chain (i.e., C-25) (15,22).

The dynamics of membrane proteins play an important role in cellular functions. Fluidity of the lipid matrix appears to be a major mechanism whereby the dynamics of membrane proteins are controlled (23,24). Thus, the function of membrane carriers, receptors and enzymes can be markedly affected by changes in lipid microviscosity (25–28). Borochov and Shinitzky (29) have shown that cholesterol depletion, which reduces membrane microviscosity, resulted in masking of membrane proteins and, conversely, an increase in membrane microviscosity, through cholesterol enrichment in red cell membranes resulted in an increase of protein exposure to the aqueous surrounding. Insertion of OS into the red cell membrane has also been shown to alter lipid fluidity and protein conformation (15).

Although the inhibition of HMG–CoA reductase by OS as a mechanism of OS-induced modulation of cell functions has been extensively investigated, relatively little attention has been directed to the possible effects of OS on the membrane physical properties (6,13). OS, being cholesterol analogues, are readily incorporated into lipid bilayers (13) and thus affect the structural and functional integrity of the mammalian plasma membrane (15). Under *in vivo* conditions, OS may accumulate in the plasma membranes of T cells, which have a long life span, resulting in cellular dysfunction and leading to immunosuppression. This would be expected to occur particularly when the organism is exposed to increased oxidant stress. The alteration of cell membrane structure and the inhibition of cholesterol biosynthesis by OS may both play a role in the pathogenesis of OS-induced immunosuppression *in vivo*.

In conclusion, the results show that C-5 and C-6 oxidation products of cholesterol inhibit CTL activity, and the

C-25 oxidation product does not inhibit CTL under the short-term *in vitro* exposure conditions used in these experiments. Our results suggest that the observed inhibitory effects of OS on CTL lytic function may be partly due to modulation of lymphocyte plasma membrane fluidity and structure induced by insertion of OS into the membrane. The position of the oxygen group(s) on the OS molecules appear to influence the degree of their effect on membrane physical and functional properties.

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Sterols of Eustigmatophytes

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The oyster cannot synthesize sterols from smaller molecules but must obtain them from its diet, which consists of detritus and small organisms, i.e., mostly single-celled algae. Algae differ widely in their effectiveness as oyster food. Small (<5 μm) algae which are abundant in sterols and polyunsaturated fatty acids appear to be most effective. Recent studies have shown the occurrence of cholesterol in strains of the unicellular algae *Tetraselmis, Chaetoceros* and *Skeletonema,* sometimes in large quantities. In the study reported here, six isolates of a recently constructed algal class, the Eustigmatophyceae, have been examined for sterols and fatty acids by gas chromatography and gas chromatography/mass spectrometry. All strains were shown to contain cholesterol as the principal sterol. Two isolates contained large amounts of total sterol (400–1000 fg/cell), and one (Sticho 0–18) also contained large amounts of eicosapentaenoic acid (20:5n-3). These biochemical characteristics are desirable in a potential food source for oysters.
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The need to improve the productivity of the eastern United States oyster fisheries and our capabilities in oyster aquaculture has stimulated research into finding an optimal diet for the oyster (1,2). Most oyster diets are composed of single-celled algae, but algae differ widely in their ability to support oyster growth (2). Although representatives of most unicellular algal groups have been examined for their ability to support oyster growth, the Eustigmatophyceae has not been systematically evaluated as oyster food. This class was first proposed by Hibberd and Leedale (3) who differentiated it from other algal taxa chiefly based on ultrastructural features. Possession of unique pigment characteristics, i.e., chlorophyll a as the only chlorophyll pigment and violaxanthin as the dominant xanthophyll, was later ascribed to the class (4). As most eustigmatophycean vegetative stages are small (1–5 μm) and nonmotile unicells, identification beyond the class level is difficult, and eustigmatophytes often have been misidentified as xanthophytes or chlorophytes. Established cultures can be assigned to the Eustigmatophyceae by the absence of chlorophyll b and c and the presence of violaxanthin and/or vaucherioxanthin as the main accessory pigments. The strains analyzed in the present study were determined to be eustigmatophytes by these criteria, although all had been misidentified previously in several chlorophyte genera. Strain designations used are those assigned by the isolator.

There is little information on the effectiveness of eustigmatophytes as bivalve feed. *Nannochloropsis salinia* (strain Sticho-GSB) has been used successfully to rear larvae of the hard clam, *Mercenaria mercenaria,* through metamorphosis (Wikfors, G.H., unpublished data). One marine eustigmatophyte, *N. oculata* ("marine chlorella"), is widely used in fish aquaculture as a feed for rotifers and brine shrimp, which are subsequently given as live prey to larval marine fish (5). Recent studies have shown that oyster growth is positively correlated with several biochemical components of its diet (1,6), with sterols and polyunsaturated fatty acids being of major importance. As all available data indicate that the oyster is unable to synthesize cholesterol (its major sterol) (7) or sufficient amounts of polyunsaturated fatty acids to meet metabolic demands (8,9), algal strains containing cholesterol (or closely related sterols) and polyunsaturated fatty acids offer excellent potential as oyster food. Therefore, our recent work has involved examination of numerous phytoplanktons for their sterol and fatty acid compositions (10,11).

Only five isolates of Eustigmatophytes have been examined for sterol and fatty acid composition (12,13). Suen *et al.* (12) reported the sterol of *Nannochloropsis* sp. QII to be cholesterol, and the major polyunsaturated fatty acid to be 20:4. Volkman *et al.* (13) examined four eustigmatophyte isolates and found 20:5n-3 to be the major polyunsaturated fatty acid and cholesterol the major sterol in each isolate. The present paper reports on the sterol composition and on the major fatty acids in six isolates of the Eustigmatophyceae.

MATERIALS AND METHODS

Eustigmatophyte cultures analyzed were from the Milford Microalgal Culture Collection; strain designations and sources are given in Table 1. Most isolates are unidentified, or had been misidentified as various chlorophyte taxae; they were shown to be eustigmatophytes by spectrophotometric pigment analysis (14), which revealed that chlorophyll a was the sole chlorophyll (Table 2). High-performance liquid chromatography of xanthophyll pigments (15) confirmed the presence of violaxanthin and the absence of neoxanthin, antheraxauthin and lutein, demonstrating that each of these isolates is a eustigmatophyte. All cultures were axenic. Biomass for sterol analysis was provided by 1500 mL E medium (16) cultures in 2800-mL Fernbach flasks. Flasks were inoculated with late-log cultures, incubated at 20°C with a 12 h light/dark cycle (600 $\mu E\ m^{-2}\ s^{-1}$), and harvested, after 30 d growth, in a stationary phase characterized as N-limited (Wikfors, G.H., unpublished data). Harvested cells were concentrated by cold (5°C) centrifugation (2000 × *g*), washed with sterile NaCl solution isotonic to the growth medium, transferred to glass lyophilizer vials, frozen in a cold (–25°C) methanol bath, and lyophilized with a Virtis Unitrap 100 Freeze-dryer (American Optical Company, Buffalo, NY).

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Abbreviations: GC, gas chromatography; MS, mass spectrometry.

TABLE 1

Sources of Eustigmatophycean Algae Analyzed

Strain	Taxon	Shape	Size (μm) mean (±SE)	Isolator	Location isolated
Sticho-GSB	*Nannochloropsis salina*	Ovoid	6.37 ± 0.13 × 3.79 ± 0.07	Ryther	Great South Bay, NY
U-106	Unidentified	Sphere	2.65 ± 0.09	Ukeles	Milford, CT (estuarine)
U-112	Unidentified	Sphere	3.34 ± 0.08	Ukeles	Milford, CT (estuarine)
Sticho-M	*Nannochloropsis* sp.	Sphere	2.95 ± 0.09	Guillard	Milford, CT (estuarine)
U-113C	Unidentified	Sphere	3.16 ± 0.08	Ukeles	Milford, CT (estuarine)
Sticho 0–18	Unidentified	Ovoid	4.67 ± 0.15 × 2.33 ± 0.06	Guillard	Martha's Vineyard, MA (coastal pond)

TABLE 2

Pigment Ratios in Eustigmatophycean Algae Analyzed

	Chlorophyll a	Chlorophyll b	Chlorophyll c	Total carotenoids
Sticho-GSBc	1.00:	0.03:	0.02:	0.54
U-106	1.00:	0.03:	0.00:	0.34
U-112	1.00:	0.03:	0.02:	0.50
Sticho-M	1.00:	0.04:	0.05:	0.45
U-113C	1.00:	0.02:	0.01:	0.26
Sticho 0–18	1.00:	0.04:	0.04:	0.26
580[a]	1.00:	0.34:	0.02:	0.35

[a]*Chlorella autotrophica*, strain 580, is included for comparison of chlorophyte pigment composition; ratio of A/B or A/C < 0.1 is not significantly different from 0.

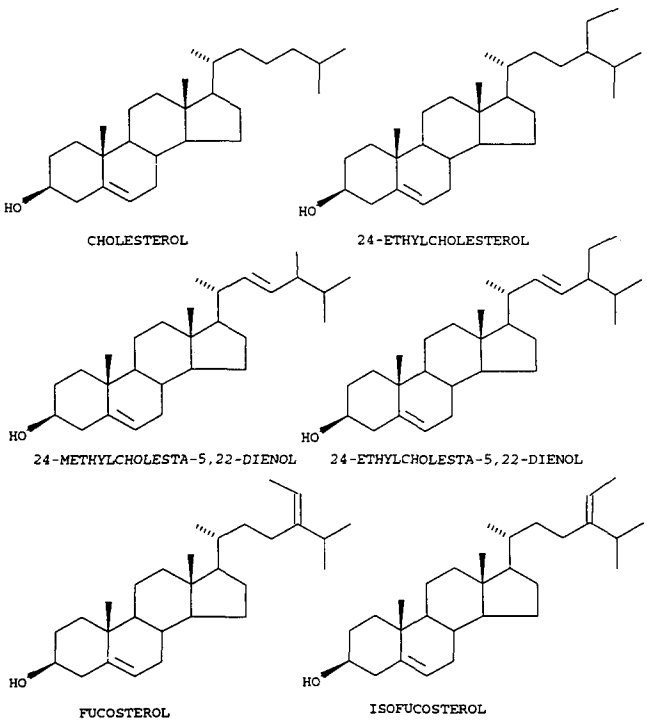
The number of cells contained within each lyophilizer vial was determined microscopically with an Improved Neubauer Hemocytometer (American Optical Company) containing a subsample of a volumetrically diluted cell concentrate. For dry weight measurements, cells were collected on a glass-fiber filter, rinsed with isotonic ammonium formate solution, dried for 8 h at 80°C, and weighed.

Lyophilized samples were extracted overnight in a Soxhlet with chloroform/methanol (2:1, vol/vol). The form of sterols present in the phytoplankton was determined by fractionating the total lipids on a Biosil A column. Free sterols were generated from esters and glycosides (17), followed by sterol identification and quantitative analysis by capillary gas chromatography (GC) on SPB-1 (18). Sterols were identified by comparing their relative retention times vs. those of cholesterol in capillary GC with those of authentic standards. Identifications were confirmed by GC/mass spectrometry (GC/MS) (18). Major fatty acids (over 10% of total) were identified by capillary GC on a 0.25 mm × 100 m column coated with a 0.2 μm film of SPB-2560.

RESULTS AND DISCUSSION

The major sterol in all Eustigmatophyte isolates was cholesterol, with amounts ranging from 52–80% of total sterol. Smaller amounts of 24-ethylcholesterol and isofucosterol were also present in many of the isolates (Scheme 1, Table 3). From their sterol compositions, four isolates (Sticho-GSB, U-106, U-112 and Sticho-M) were essentially indistinguishable from each other, having a total sterol content of 13–30 fg/cell. Strain U-113C had high total sterol content (480 fg/cell) with 79% of the total sterol being present as cholesterol. Isolate Sticho 0–18 was distinguishable from all the other isolates by its higher dry weight (26 pg/cell), its very high total sterol content (1000 fg/cell), and by the fact that the companion sterols of cholesterol were 24-methylcholesta-5,22-dienol and 24-ethylcholesta-5,22-dienol (Scheme 1). GC/MS data and GC relative retention times of the sterols identified were identical to those of authentic standards. Although most algal sterols with asymmetry at C-24 have 24β alkyl groups (9), the C-24 stereochemistry was not determined in this work. The sterols in all six isolates were present almost exclusively as free sterols. Steryl esters and steryl glycosides were present at less than 5% of total sterol.

The major fatty acid of the strains Sticho-GSB, U-106, U-112 and U-113C was palmitic acid (16:0); only small



SCHEME 1

TABLE 3

Sterols of Eustigmatophytes

Isolate	Cellular dry wt.[a] (pg/cell)	Sterol composition						
		fg/cell	CHOL[b]	MC5,22	EC5,22	EC	FUCO	ISOFUCO
Sticho-GSB	9.85(0.08)	21	52			39		5
U-106	6.50(0.23)	30	56			35		2
U-112	5.58(0.14)	20	52			43		5
Sticho-M	6.36(0.08)	13	56			33		5
U-113C	13.90(0.26)	480	79				15	2
Sticho 0–18	25.77(0.86)	1000	80	13	4			

[a]Mean of four determinations (SE).
[b]As % of total sterol; CHOL, cholesterol; MC5,22, 24-methyl-cholesta-5,22-dienol; EC5,22, 24-ethylcholesta-5,22-dienol; EC, 24-ethylcholesterol; FUCO, fucosterol; ISOFUCO, isofucosterol.

quantities (less than 10% of total fatty acid) of long-chain polyunsaturated fatty acids were present (Table 4). Isolates of Sticho-M and Sticho 0–18 contained eicosapentaenoic acid (20:5n-3) as the major fatty acid. Thus, Sticho-M can be differentiated from the other three strains with similar sterol compositions, which have 16:0 rather than 20:5 as the major fatty acid. Biochemical similarities among strains U-106, U-112 and Sticho-GSB suggest that all these strains may be isolates of *N. salinia.*

Interestingly, Suen *et al.* (12) detected 20:4, but not 20:5n-3 or 22:6n-3, in *Nannochloropsis* sp. II, but all strains examined by the Volkman group (19,20) had large amounts of 20:5n-3. None of the isolates analyzed in our study had both sterol and fatty acid compositions consistent with *N. oculata,* the species frequently used in aquaculture (5).

Cholesterol is the major sterol in all eustigmatophytes studied in this and in previous work, although the quantity of cholesterol varies greatly among isolates. About half the strains appear rich in long-chain polyunsaturated fatty acids (12,13). The early work of Dupuy (21) had shown that oysters can be grown on a mixed diet, which included one eustigmatophyte. Because Sticho 0–18 is rich in 20:5n-3 and very high in sterol content, it appears to have the greatest potential as oyster food of the eustigmatophyte isolates studied to date.

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TABLE 4

A Comparison Sterol and Fatty Acid Compositions of Eustigmatophytes

Strain	CHOL[b] (fg/cell)	Minor sterols[a]				Major fatty acids (% of total)				
		EC	FUCO	ISOFUCO	MC5,22	16:0	16:1	18:1	18:3	20:5
Sticho-GSB	11	X				59	4	1	—	—
U-106	17	X				38	1	18	—	2
U-112	10	X				35	20	4	—	2
Sticho-M	7	X				17	28	5	—	29
U-113C	480		X			53	20	3	—	3
Sticho 0–18	800				X	19	11	12	1	25
Nannochloropsis oculata[c] CS-216	34		X	X		18	27	9	1	28
N. oculata[c] CS-179	15		X	X		14	29	7	4	29
CS-246[c]	22		X	X		20	21	5	2	33
N. salina[c] CS-190	27	X		X		28	32	9	1	16
Nannochloropsis sp. QII[d]						22	27	11	1	26[e]

[a]Over 5% of total sterol. [b]Abbreviations as in Table 3. [c]From References 15 and 18. [d]From Reference 12, only cholesterol, quantity not reported. [e]Identified as 20:4 in this strain.

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ARTICLE

β-Carotene Transport in Human Lipoproteins. Comparisons with a-Tocopherol

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The purpose of this study was to investigate the temporal relationships of the transport of β-carotene in human lipoproteins. We administered 60 mg β-carotene with breakfast to nine fasting subjects, then blood samples were collected at intervals of up to 75 h, lipoproteins were isolated, and β-carotene was quantitated. β-Carotene concentrations in chylomicrons and very low density lipoproteins (VLDL) peaked at 6 and 9 h, respectively. Nonetheless, at all time points the majority of plasma β-carotene was contained in low density lipoproteins (LDL), while high density lipoproteins (HDL) carried a smaller portion (at 24 h, 73 ± 8% in LDL as compared with 23 ± 5% in HDL). In three subjects, transport of β-carotene was compared with the results of earlier studies on the transport of stereoisomers of α-tocopherol. Unlike plasma *RRR*-α-tocopherol concentrations, which are maintained by the preferential incorporation of *RRR*-α-tocopherol into VLDL by the liver, β-carotene increased and decreased in VLDL similarly to *SRR*-α-tocopherol, a stereoisomer whose concentrations are not maintained in plasma. In conclusion, β-carotene is primarily transported in the plasma in LDL, but its incorporation by the liver into lipoproteins does not appear to be enhanced.

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In intestinal cells, β-carotene can either be secreted directly in chylomicrons, or it can be converted by intestinal cells to retinol, which is esterified with palmitic acid for export in chylomicrons (1). For the purpose of calculating the recommended dietary allowance for vitamin A, it has been assumed that 33% of a dose of β-carotene is absorbed, and 50% is converted to retinol (2). Most (75–79%) of the plasma carotenoids are associated with low density lipoproteins (LDL), with the remainder being carried by high density lipoproteins (HDL) (3–7).

We have been studying the transport of α-tocopherol in lipoproteins using deuterium-labeled tocopherols, both α- and γ-tocopherols, as well as two stereoisomers of α-tocopherol (*RRR*- and *SRR*-) (8–12). These studies have demonstrated that there is little discrimination between tocopherols during absorption and secretion in chylomicrons, but, subsequently, there is a preferential incorporation of *RRR*-α-tocopherol, the naturally occurring stereoisomer, into very low density lipoproteins (VLDL) (13). During catabolism of the triglyceride-rich lipoproteins (both chylomicrons and VLDL), there is rapid transfer of tocopherols between the lipoproteins.

β-Carotene is more hydrophobic than α-tocopherol; therefore, it may not be transferred to lipoproteins during chylomicron catabolism in a manner similar to tocopherols. Furthermore, available evidence suggests that a tocopherol binding protein is required for the preferential incorporation of *RRR*-α-tocopherol into nascent VLDL (9,14). Although a hepatic carotenoid binding protein, which could be involved in the incorporation of β-carotene into VLDL, has been described in a preliminary report by Okoh and Lakshman (15), β-carotene may behave more like γ- or *SRR*-α-tocopherols, which are not efficiently resecreted in VLDL into the plasma (16). The purpose of this study was to evaluate the temporal relationships of the transport of β-carotene in human plasma lipoproteins.

EXPERIMENTAL DESIGN AND METHODS

Experimental protocols. This study was carried out within the guidelines of the Institutional Review Board of New York University Medical Center (New York, NY). Ten subjects gave written informed consent and had no abnormalities of lipid or lipoprotein metabolism. The subjects were instructed to consume a gelatin capsule containing 60 mg β-carotene beadlets [Hoffman La Roche, Nutley, NJ; beadlets are a formulation of β-carotene that are especially bioavailable (17)] following an overnight fast, along with a suggested breakfast consisting of 1 cup whole milk, 1 cup vanilla ice cream, 1 banana, 1 serving of cereal (Cheerios) and 1 cup of coffee. Subsequent meals were the subject's choice, but the subjects were instructed to avoid carrots, yellow squash, pumpkin, broccoli and spinach a week before the study as well as during the course of the study. The characteristics of the nine subjects reported here are shown in Table 1.

Blood samples (15 mL/time point) were collected in ethylenediaminetetraacetic acid (EDTA) tubes (Becton-Dickinson, Rutherford, NJ) at 0, 3, 6, 9, 11, 28, 53 and 76 h after the subjects consumed the β-carotene. Plasma was separated from blood cells by centrifugation; a 1.0 mL aliquot of plasma was used for β-carotene quantitation, and the remainder was used for isolation of the lipoprotein fractions. Samples were protected from light at all times.

Lipoprotein isolation. Chylomicrons and lipoproteins were isolated as described previously (8). Briefly, chylomicrons were isolated from duplicate samples of 4 mL plasma overlayed with 2 mL saline (0.15 M NaCl, 0.3 mM EDTA, pH 7.4) by centrifugation for 8 min at 40,000 rpm

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Abbreviations: AUC, areas under the plasma curves; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

TABLE 1

Characteristics of Study Subjects

Subject	Sex	Triglycerides (mmol/L)	Cholesterol (mmol/L)	HDL[a] cholesterol (mmol/L)
1	M	0.46	4.29	1.40
2	F	0.67	4.91	1.99
3	M	1.21	4.55	0.83
4	F	0.73	4.22	ND[b]
5	F	0.79	3.36	ND
6	M	0.36	4.73	1.76
7	F	0.99	4.58	1.50
8	F	0.58	4.86	ND
9	M	0.54	4.01	1.11
Mean ± SD		0.70 ± 0.27	4.39 ± 0.49	1.43 ± 0.42

[a]HDL, high density lipoproteins. [b]ND, not determined.

using a swinging bucket rotor (TLS 55) and a TL 100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The remaining lipoprotein fractions were isolated in two sequential steps by ultracentrifugation for 2 h at 435,680 × *g* using a fixed angle rotor (TLA 100.2) with density changes achieved by adding solid KBr. The density ranges used were: VLDL d < 1.006, LDL 1.006 < d < 1.063 and HDL 1.063 < d < 1.21.

β-Carotene analysis. β-Carotene was analyzed using a modification of the method of Stacewicz-Sapuntzakis *et al.* (18). Briefly, upon isolation, the lipoprotein fractions and the plasma samples were saponified with alcoholic potassium hydroxide in the presence of 1% ascorbic acid, then were extracted with hexane, and an aliquot of the extract was dried under nitrogen. The samples were resuspended in 50 μL chloroform and diluted to 200 μL with mobile phase (methanol/acetonitrile/tetrahydrofuran, 50:45:5, by vol) containing 100 mg ammonium acetate per L of mixture. Aliquots containing between 1 and 50 mg β-carotene were injected into a high-pressure liquid chromatography system (Waters, Milford, MA) equipped with a C18 column (Waters) at a flow rate of mobile phase of 2 mL/min, and detected at 450 nm using a Waters variable wavelength ultraviolet detector. β-Carotene is not stable enough to use purified standards for daily use as calibrators. Therefore, β-carotene for use as a standard was extracted daily from beadlets (a stabilized form with added antioxidants). β-Carotene in plasma or lipoproteins was quantitated by comparison of peak areas with those of a β-carotene standard.

Deuterated tocopherol study. Three of the study subjects (numbers 2, 3 and 4) had previously participated in studies using deuterated tocopherols (12); results of various concentrations of deuterated α-tocopherols are reported here for comparison with the β-carotene values. Briefly, each subject consumed a capsule containing 50 mg each of d_6-*RRR*-α-tocopheryl acetate, d_3-*SRR*-α-tocopheryl acetate and d_2-*RRR*-γ-tocopherol, with a breakfast following an overnight fast. Blood samples were drawn into EDTA tubes at approximately 0, 3, 6, 9, 12, 27, 51 and 75 h after consumption of the tocopherols. Subjects were allowed to eat *ad libitum*.

Plasma was immediately separated from blood cells by centrifugation. An aliquot (1.0 mL) was frozen for subsequent tocopherol analysis, and the remainder was used for isolation of the lipoprotein fractions, as described in a previous paragraph. Frozen plasma and lipoprotein fractions were shipped on dry ice to the National Research Council of Canada where they were kept frozen at –80°C until analyzed. The amounts of d_2-γ-tocopherol, d_3-, d_6-, d_9-α-tocopherols and unlabeled (d_0-)α- and γ-tocopherols in the collected tocopherol fraction were determined by gas chromatography/mass spectrometry after conversion to their trimethylsilyl ethers. The absolute concentrations of d_0-, d_3- and d_6-α-tocopherols and d_0- and d_2-γ-tocopherols in the plasma and lipoprotein samples were obtained by comparing their respective peak areas with the peak areas of the corresponding d_9-α- and d_{17}-γ-tocopherols added (8,9,19).

Data analysis and statistical methods. Calculations were carried out using a Macintosh II computer (Apple Computers, Cupertino, CA). The concentrations of lipoprotein β-carotene were summed and compared with the plasma concentrations of β-carotene for each time point. The total lipoprotein concentrations ranged from 80–120% of the plasma concentrations. The plasma concentration was used as the total amount of β-carotene, and the sum of the lipoprotein β-carotene was corrected to this value. The lipoprotein β-carotene distribution was based on the actual measurements. The mean ± standard deviations were calculated for each time point for the plasma and each of the lipoprotein fractions for the nine subjects. The area under the curve (AUC) was calculated for the plasma carotenoids by summing the trapezoids estimated from the X–Y data points using KaleidaGraph (Synergy Software, PCS Inc., Reading, PA). The statistical significance of the differences was determined using the statistical analysis program, Super Anova (Abacus Concepts, Berkeley, CA), using analysis of variance and least square means comparisons. Results of the statistical tests were considered to be significant at the 95% confidence level ($P < 0.05$).

RESULTS

The concentrations of β-carotene in the plasma and lipoproteins from nine subjects are shown in Figure 1. One of the ten subjects studied had almost undetectable plasma β-carotene levels; his data are not included. Previously, Prince and Frisoli (20) reported that dietary fat is necessary for β-carotene absorption; likely the omitted subject did not consume breakfast with the dose. Plasma β-carotene of the other nine subjects increased up to 27 h and then remained relatively constant for the remainder of the study. The AUC ranged from 8 to 116 nmol/mL · h with a mean ± standard deviation of 51 ± 33 nmol/mL · h. Despite the wide range of β-carotene concentrations, in all subjects β-carotene was observed to peak first in the chylomicrons, and then in the VLDL (Fig. 1). HDL β-carotene concentrations appeared to increase only during triglyceride-rich lipoprotein catabolism (up to 11 h) and then reached a plateau, whereas LDL β-carotene concentrations increased for a longer period of time (up to 48 h).

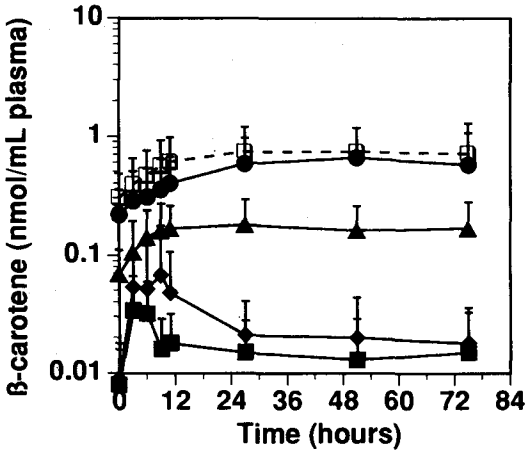

FIG. 1. Plasma and lipoprotein β-carotene concentrations. Following an overnight fast, nine subjects each consumed a gelatin capsule containing 60 mg β-carotene beadlets (Hoffmann La Roche, Nutley, NJ) along with breakfast. Blood samples (15 mL/time point) were collected in EDTA tubes (Becton-Dickinson, Rutherford, NJ) at 0, 3, 6, 9, 11, 28, 53 and 76 hours. Plasma was separated from blood cells by centrifugation; chylomicrons and lipoprotein fractions were isolated as described in the Experimental Design and Methods section. The density ranges of the lipoproteins were: very low density (VLDL) d < 1.006, low density (LDL) 1.006 < d < 1.063, and high density (HDL) 1.063 < d < 1.21. Shown are means + SD of the β-carotene concentrations in plasma and each of the lipoprotein fractions. Key: ❑, plasma; ●, LDL; ▲, HDL; ◆, VLDL; ■, chylomicrons.

The percentage distribution of plasma β-carotene in the lipoprotein fractions throughout the study are shown in Figure 2. Early on, increases in proportion of plasma β-carotene were observed in the triglyceride rich-lipoproteins (in chylomicrons a statistically significant increase compared to baseline was observed at 3 h, $P < 0.001$), while the VLDL showed significant increases at 3 h ($P < 0.001$), 6 h ($P < 0.01$) and 9 h ($P < 0.01$). During triglyceride-rich lipoprotein catabolism from 0 to 9 h, HDL β-carotene increased 6% (22 ± 5 to 28 ± 8%, $P < 0.03$). At the outset of the study, 72 ± 6% of the plasma β-carotene was found in LDL, which contained most of the plasma β-carotene at all time points. However, because the other lipoproteins transported β-carotene during the initial part of the study, the proportion of plasma β-carotene in LDL decreased from 3 to 11 h and significantly smaller fractions of plasma β-carotene were present in LDL at 3 h ($P < 0.01$), 6 h ($P < 0.001$), 9 h ($P < 0.01$) and 11 h ($P < 0.05$). By 27 h, the distribution of β-carotene in all lipoprotein fractions had returned to baseline values.

Three of the subjects (numbers 2, 3 and 4) studied had previously participated in studies using deuterated α-tocopherols (12). For purposes of comparison of α-tocopherol with β-carotene (because β-carotene was unlabeled), the increase in β-carotene concentrations is compared with the deuterated α-tocopherol concentrations. As shown in Figure 3, the plasma concentrations of both labeled stereoisomers of α-tocopherol were about fivefold greater than those of β-carotene at 3 h. By 24 h, the concentrations of β-carotene reached those of *SRR*-α-tocopherol. Subsequently, the concentrations of β-carotene and *SRR*-α-tocopherol decreased at a similar rate.

Examination of the lipoprotein patterns of β-carotene and of the α-tocopherols in these three subjects shows marked differences in the transport of β-carotene and α-tocopherol (Fig. 4). It appears that the peak in chylomicron β-carotene precedes that of α-tocopherol; however, because the data are from separate studies, the temporal relationship between the chylomicron transport of α-tocopherol and β-carotene cannot be stated with certainty. In

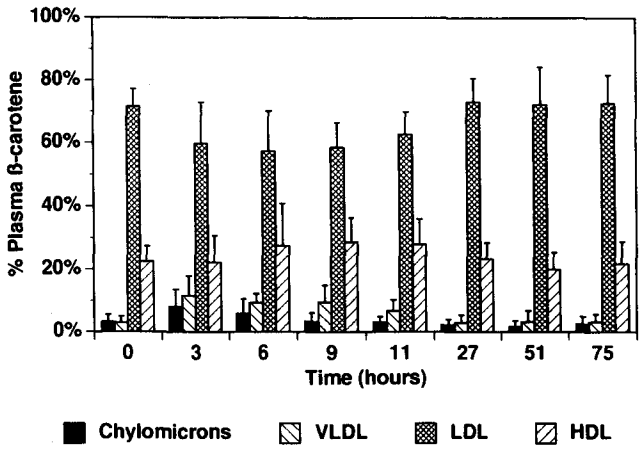

FIG. 2. Percentage distribution of β-carotene in lipoprotein fractions. The percentage distribution of plasma β-carotene in each of the lipoprotein fractions described in Figure 1 was calculated for each subject. Shown are the means + SD for each fraction. Abbreviations as in Figure 1.

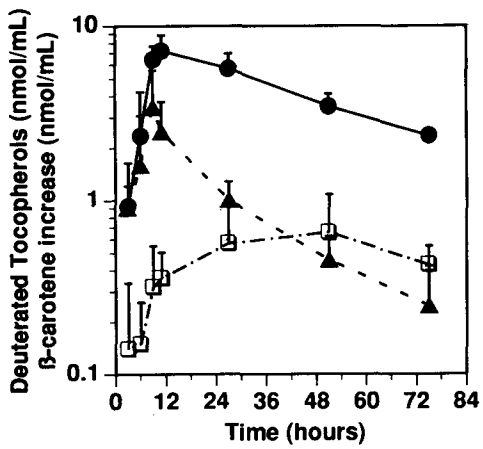

FIG. 3. Comparisons of plasma concentrations of labeled α-tocopherol stereoisomers with increases in β-carotene concentrations. Three subjects (numbers 2, 3 and 4) had previously participated in a study on the transport of vitamin E by consuming a dose containing 50 mg of each *RRR*- and *SRR*-α-tocopherols labeled with 6 or 3 atoms of deuterium, respectively (12). The concentrations of β-carotene in the plasma of these subjects in response to the dose of β-carotene beadlets were estimated by subtracting the concentration of β-carotene at time 0. Shown are the means + SD of the concentrations of deuterated tocopherols (*RRR*-α-tocopherol and *SRR*-α-tocopherol) and the increases in β-carotene above background. Key: ▲, d_3-*SRR*; ●, d_6-*RRR*; ❑, β-carotene increase.

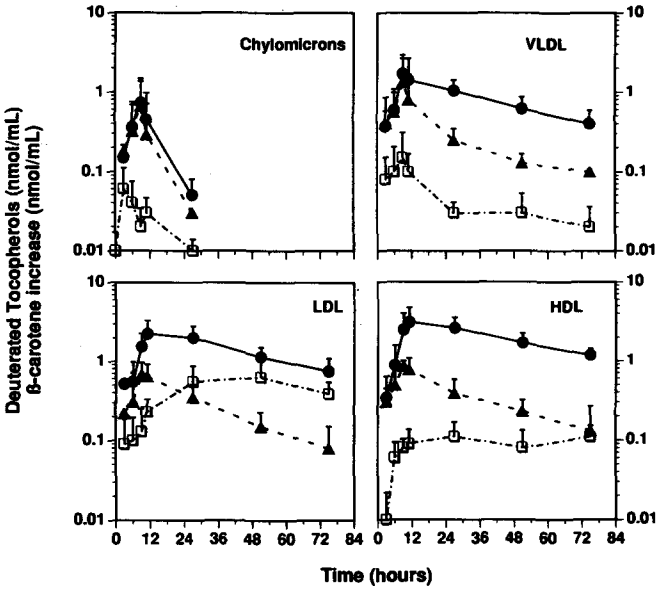

FIG. 4. Lipoprotein concentrations of β-carotene increases and labeled stereoisomers of α-tocopherol. Lipoprotein (as described in Figure 1) concentrations of deuterium-labeled *stereoisomers of α-tocopherol* and *β-carotene increases* are shown from the three subjects described in Figure 3. Key as in Figure 3; abbreviations as in Figure 1.

VLDL, β-carotene concentrations (although fivefold lower) parallel those of *SRR*-α-tocopherol. In LDL, the concentrations of β-carotene increased, nearly reaching those of *RRR*-α-tocopherol at 48 h, unlike those of *SRR*-α-tocopherol, which began to decrease at 9 h. In HDL, β-carotene concentrations essentially reach their maxima by 11 h.

DISCUSSION

This study demonstrates that β-carotene is absorbed and secreted by the intestine in chylomicrons and is subsequently secreted by the liver in VLDL. Increases in HDL β-carotene were observed during the catabolism of these two triglyceride-rich lipoproteins in the plasma. Unlike HDL β-carotene, which increased only during the first 12 h of the study, LDL β-carotene transport in LDL concentrations increased up to 48 h. These increases are likely the result of the conversion of VLDL to LDL.

The comparisons between the transport of α-tocopherol and β-carotene provide further insights in the transport of these two hydrophobic nutrients. Similar amounts of deuterated α-tocopherols and unlabeled β-carotene were administered, but increases in plasma concentrations of β-carotene were almost tenfold lower (Fig. 3). It is likely that the lower plasma concentrations of β-carotene result from a combination of low absorption rates and low plasma incorporation. With respect to absorption, although both β-carotene and α-tocopherols are transported in chylomicrons, the greater hydrophobicity of β-carotene may limit its intracellular transport and thus its incorporation into chylomicrons. Additionally, some β-carotene is converted to retinol and is absorbed, as retinyl palmitate (1).

With respect to plasma transport, we have previously demonstrated that *RRR*-α-tocopherol is preferentially enriched in plasma by 24 h following an oral dose containing various labeled tocopherols (10,12). The preference for incorporation of *RRR*-α-tocopherol into nascent VLDL by the liver (11) likely results from the selection of this form of vitamin E by the tocopherol transfer protein (21–24). The catabolism of VLDL enriched in *RRR*-α-tocopherol in the plasma and the continued preferential secretion of *RRR*-α-tocopherol in VLDL by the liver are probably responsible for maintaining plasma α-tocopherol concentrations (13,25). In VLDL, β-carotene concentrations paralleled those of *SRR*-α-tocopherol, suggesting that β-carotene is incorporated into VLDL in a nonspecific manner. Further, the differences in the α-tocopherol and β-carotene concentration curves in LDL and HDL demonstrate differences in transport of these two nutrients. Because of its hydrophobicity, β-carotene is likely to be located in the hydrophobic lipoprotein core, which would be consistent with the preference of β-carotene for LDL rather than those for HDL. In contrast, HDL β-carotene increased during the first few hours of the experiment, suggesting that during the transfer of surface components from chylomicrons to HDL there is also transfer of some core lipids. Furthermore, HDL β-carotene represents a relatively small fraction of β-carotene, suggesting that β-carotene does not readily exchange between lipoproteins *in vivo* or *in vitro* (26).

In conclusion, this study demonstrates that the plasma and lipoprotein transport of two hydrophobic nutrients, β-carotene and *RRR*-α-tocopherol, are dissimilar. β-Carotene does not readily exchange between lipoproteins, and it appears to concentrate in LDL, whereas *RRR*-α-tocopherol is present in all of the lipoprotein fractions. It has been suggested that β-carotene might function as an antioxidant and protect LDL from peroxidative damage during oxidative stress. However, recent studies have suggested that β-carotene supplementation does not necessarily increase the resistance to oxidative stress (6). Thus, the beneficial effects of elevated LDL β-carotene remain to be demonstrated.

ACKNOWLEDGMENTS

David Fry and Nora Lagmay provided excellent technical assistance. This study was supported in part by grants from the U.S. public Health Service, HL 30842, and from Hoffmann La Roche, which also supplied β-carotene beadlets.

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Comparison of Fatty Acid α-Oxidation by Rat Hepatocytes and by Liver Microsomes Fortified with NADPH, Fe^{3+} and Phosphate

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Rat liver microsomes, when fortified with NADPH, Fe^{3+} and phosphate, can catalyze the oxidative decarboxylation (α-oxidation) of 3-methyl-substituted fatty acids (phytanic and 3-methylheptadecanoic acids) at rates that equal 60–70% of those observed in isolated hepatocytes (Huang, S., Van Veldhoven, P.P., Vanhoutte, F., Parmentier, G., Eyssen, H.J., and Mannaerts, G.P., 1992, *Arch. Biochem. Biophys. 296*, 214–223). In the present study we set out to identify and compare the products and possible intermediates of α-oxidation formed in rat hepatocytes and by rat liver microsomes. In the presence of NADPH, Fe^{3+} and phosphate, microsomes decarboxylated not only 3-methyl fatty acids but also 2-methyl fatty acids and even straight chain fatty acids. The decarboxylation products of 3-methylheptadecanoic and palmitic acids were purified by high-performance liquid chromatography and identified by gas chromatography/mass spectrometry as 2-methylhexadecanoic and pentadecanoic acids, respectively. Inclusion in the incubation mixtures of glutathione plus glutathione peroxidase inhibited decarboxylation by more than 90%, suggesting that a 2-hydroperoxy fatty acid is formed as a possible intermediate. However, we have not yet been able to unequivocally identify this intermediate. Instead, several possible rearrangement metabolites were identified. In isolated rat hepatocytes incubated with 3-methylheptadecanoic acid, the formation of the decarboxylation product, 2-methylhexadecanoic acid, was demonstrated, but no accumulation of putative intermediates or rearrangement products was observed. Our data do not allow us to draw conclusions on whether the reconstituted microsomal system is representative of the cellular α-oxidation system. However, the results we obtained with [3-^{3}H]-labelled fatty acids indicate that during α-oxidation in intact cells the hydrogen at carbon-3, which carries the methyl branch, is not attacked.

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is an isoprenoid-derived fatty acid that accumulates in the body fluids and tissues of patients suffering from Refsum's disease, a rare inherited metabolic disorder affecting the nervous system (1). Due to the presence of a methyl group at carbon-3, phytanic acid cannot be degraded *via* the classical β-oxidation pathway. Instead, it is first oxidatively decarboxylated by α-oxidation, yielding CO_2 and a 2-methyl-substituted fatty acid, pristanic acid (1), which can then be degraded *via* peroxisomal β-oxidation (2).

The mechanism of phytanic acid α-oxidation has remained elusive for many years. Earlier studies, by Steinberg's group and others (3–7), had suggested that phytanic acid α-oxidation involved an unusual α-hydroxylation step to yield 2-hydroxyphytanic acid as an intermediate and that the enzymatic activity was located in the mitochondria. In recent years, there have been a number of reports (8–12) that showed that phytanic acid accumulation also occurs in the body fluids and tissues of patients with general peroxisomal disorders, e.g., Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy and rhizomelic chondrodysplasia punctata, suggesting that peroxisomes may play a role in phytanic acid α-oxidation. However, cell fractionation studies have produced contradictory results—the highest α-oxidation activity was found in either mitochondrial (7,13,14) or combined mitochondrial plus cytosolic fractions (15). Very recently, Wanders and van Roermund (16) suggested that α-oxidation involves the sequential action of mitochondria and peroxisomes, and Singh *et al.* (17) concluded that α-oxidation is located in mitochondria in the rat, but it is in the peroxisomes in humans. Reports on the cofactor requirements of phytanic acid α-oxidation have also been inconsistent. Tsai *et al.* (7) postulated that α–oxidation requires O_2, NADPH and Fe^{3+}, whereas others found that, besides O_2, the only cofactors necessary are adenosine triphosphate (ATP) and Mg^{2+} (13–16) or ATP, Mg^{2+} and CoA (17). A further complication in interpreting the above studies is the fact that the α-oxidation rates obtained in these broken cell systems were extremely low as compared with those obtainable in intact cells (18).

In a recent report (18), we have demonstrated that the maximal rates of α-oxidation in isolated rat hepatocytes amounted to 7 and 10 nmol/min × 10^8 cells with phytanic acid and 3-methylheptadecanoic acid, respectively. In rat liver homogenates, optimal rates of α-oxidation, which equalled 60–70% of those observed in the intact hepatocytes, required O_2, NADPH, Fe^{3+} and phosphate ions. Subcellular fractionation revealed that under our assay conditions α-oxidation is located in the endoplasmic reticulum. Using the same assay conditions, we now find that rat liver microsomes decarboxylate not only 3-methyl fatty acids but also 2-methyl fatty acids, and even straight chain fatty acids. We furthermore attempted to identify and purify the decarboxylation products and the putative reaction intermediate(s).

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Abbreviations: ATP, adenosine triphosphate; BSTFA–TMCS, *bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane; EI, electron impact; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography; TMS, trimethylsilyl.

MATERIALS AND METHODS

Chemicals. Collagenase, superoxide dismutase, catalase, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim (Heidelberg, Germany). Glutathione peroxidase, cytochrome C and metyrapone were from Sigma (St. Louis, MO). Glutathione was from Merck (Darmstadt, Germany). Palmitic acid was from Janssen Chimica (Beerse, Belgium). Phytanic acid, [1-^{14}C]phytanic acid (specific radioactivity, 54 mCi/mmol, initial radiochemical purity 99.2%), 3-methylheptadecanoic acid, 3-methyl-[1-^{14}C]heptadecanoic acid (specific radioactivity, 54 mCi/mmol, initial radiochemical purity 99.8%), 2-methylpalmitic acid and 2-methyl-[1-^{14}C]palmitic acid (specific radioactivity, 35 mCi/mmol, initial radiochemical purity 98.8%) were synthesized as described previously (2,18). [1-^{14}C]Palmitic acid (specific radioactivity, 57 mCi/mmol, stated radiochemical purity 97.7%) and [9,10-^{3}H]palmitic acid (specific radioactivity, 51.6 Ci/mol, stated radiochemical purity 98.4%) were obtained from New England Nuclear (Bad Homberg, Germany). [^{3}H]$NaBH_4$ (specific radioactivity 32 Ci/mmol) was from Amersham International (Amersham, England) and [1,2-^{13}C]palmitic acid (isotopic purity>99%) was purchased from Isotec Inc. (Miamisburg, OH). *Bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA–TMCS) was obtained from Alltech Associates (Laarne, Belgium). Ethereal diazomethane was prepared from Diazald^{R} as recommended by the manufacturer (Aldrich, Brussels, Belgium).

Synthesis of 3-methyl-[3-^{3}H]heptadecanoic acid. Tritiated 3-methylheptadecanoic acid was prepared from [2-^{3}H]hexadec-2-yl methanesulfonate, similarly to the previously described synthesis of cold 3-methylheptadecanoic acid (18). [2-^{3}H]Hexadec-2-yl methanesulfonate was obtained from 2-hexadecanol as follows. 2-Hexadecanol (245 mg, 1 mmol) was dissolved in 20 mL of hexane and oxidized by adding 35 mL of 2.7 M CrO_3/4.3 M H_2SO_4. After vigorous stirring for 30 min at room temperature, 2-hexadecanone was extracted into hexane. The hexane phase was washed twice with saturated $NaHCO_3$, thrice with water, and analyzed by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (60:40:1, by vol). Visualization by iodine or ultraviolet-illumination revealed only one spot with an R_f value of 0.82. In the next step, 2-hexadecanone (48.2 mg, 0.2 mmol, dissolved in 2 mL of tetrahydrofuran) was reduced by adding 50 μmol of [^{3}H]$NaBH_4$ (10 mCi), which was dissolved in 0.47 mL of 0.1 N NaOH. After 5 h, another aliquot of $NaBH_4$ was added (60 μmol, dissolved in 1 mL of 0.2 N NaOH), and the reaction was stirred further at room temperature (65 h in total) to ensure complete reduction. The reaction mixture was diluted to 10 mL with water, NaCl was added to a final concentration of 1 M, and the mixture was extracted thrice with diethyl ether. The combined organic phases, after washing with 0.1 M NaOH, contained 4.6 mCi of tritium. Most of label (76%) was associated with 2-hexadecanol (R_f 0.52). Without further purification, the crude 2-hexadecanol was mesylated (R_f 0.58), and then converted to diethyl-2-(1′-methylpentadecyl)malonic acid and decarboxylated (18). Following purification by preparative TLC, 3-methyl-[3-^{3}H]heptadecanoic acid (R_f 0.50) was obtained with a yield of 33% and a specific radioactivity of 6.93 mCi/mmol (radiochemical purity, 99.8%). Upon further analysis either as fatty acid or methyl ester by TLC or high-performance liquid chromatography (HPLC), no difference was observed between the tritiated compound and 3-methylheptadecanoic acid or 3-methyl-[1-^{14}C]heptadecanoic acid, which were synthesized by different routes (18).

Preparation of rat hepatocytes and liver microsomes. Male Wistar Albino rats (100–120 g body weight) were maintained on a standard laboratory diet until sacrifice. Isolated hepatocytes were prepared as described by Zahlten and Stratman (19). For the preparation of liver microsomes, livers were removed immediately after decapitation and homogenized in 0.25 M sucrose containing 5 mM 3-[*N*-morpholino]propanesulfonic acid, buffer pH 7.4. The homogenates (12.5%, wt/vol) were centrifuged at 10,000 × *g* for 24 min. The resulting postmitochondrial supernatants were then centrifuged at 100,000 × *g* for 1 h. The microsomal pellets were resuspended in the homogenization buffer and stored at –20°C. The microsomes were used within one month without significant loss of activity. Protein content was measured by the method of Peterson (20) using bovine serum albumin as standard.

Incubation of substrates and extraction of metabolic products. Incubations of radiolabelled substrates with isolated rat hepatocytes or liver microsomes and the measurement of $^{14}CO_2$ production were done essentially as described previously (18), except that the radiolabelled substrates were always purified by reverse-phase HPLC prior to the start of the incubations. In microsomal incubations, the reactions were terminated after 15 min by acidifying with 1 M HCl (final pH around 2), and the incubation mixtures were immediately transferred to extraction tubes. The fatty acids were extracted thrice with three volumes of ethyl acetate, and the combined organic extracts were dried in a stream of nitrogen and stored at –20°C before HPLC analysis. In hepatocyte incubations, the reactions were stopped by acidification with 6% (wt/vol) $HClO_4$, and the mixtures were extracted with 3.75 volumes of chloroform/methanol (1:2, vol/vol). The mixtures were left on ice for 2 h and then were centrifuged at 2,000 × *g* for 20 min at 4°C. The pellets were discarded, and the supernatants were transferred to tubes containing 1.25 volumes of water and 1.25 volumes of chloroform. After mixing, the two phases were separated by centrifugation at 2,000 × *g* for 10 min at 4°C. The upper aqueous phase was discarded, and the lower phase was dried in a stream of nitrogen. The residues were dissolved in 2 mL hexane/diethyl ether (95:5, vol/vol) and applied to a silica Sep-Pak column (9 × 20 mm, Analytichem International, Harbor City, CA). The fatty acids and the polar metabolites were eluted with 15 mL of diethyl ether. The eluates were taken to dryness under nitrogen and stored at –20°C before HPLC analysis.

Reverse-phase HPLC. The dried organic extracts were dissolved in 100 μL of methanol, and 75 μL was applied to a reverse-phase HPLC column (Nova-Pak ODS C_{18}, 4 μm, 3.9 × 150 mm; Waters, Milford, MA). The fatty acid substrates and the polar metabolites were separated with a

methanol gradient in water (containing 0.1% vol/vol, acetic acid) (see Fig. 1B), at a constant flow rate of 1 mL/min. The radioactivity was either constantly monitored with an on-line Flo-One/β HPLC radioactivity detector (Radiomatic A505; Canberra Packard, Zurich, Switzerland) or the fractions were collected and counted for radioactivity in a liquid scintillation counter. Fractions of interest were collected, taken to dryness under nitrogen, derivatized with ethereal diazomethane, and further separated by reverse-phase HPLC using the same gradient program. The radioactive fractions were collected, taken to dryness under nitrogen, and the residues were dissolved in hexane for gas chromatography/mass spectrometry (GC/MS) analysis or derivatized with BSTFA–TMCS as recommended by the manufacturer before GC/MS analysis.

Gas–liquid chromatography/MS. A Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph was directly coupled to a Finnigan MAT (San Jose, CA) TSQ70 triple quadrupole mass spectrometer. Chromatography was done on a 25 m × 0.32 mm i.d. RSL 200 chemically bonded (FSOT) fused-silica capillary column (Alltech), film thickness 0.25 μm, using splitless injection and helium as carrier gas. The column oven temperature was held at 80°C for 2 min, then increased to 150°C at a rate of 20°C/min, held at 150°C for 1 min, then increased to 200°C at a rate of 2°C/min, held at 200°C for 1 min, and then increased to 300°C at a rate of 20°C/min. The injection port and GC/MS interface were maintained at 250°C. Electron impact (EI) spectra were obtained with an ionization energy of 70 eV and a source temperature of 150°C.

RESULTS

Cofactor requirements and substrate specificity of α-oxidation. As can be seen from Table 1, in the presence of the necessary cofactors rat liver microsomes decarboxylated not only 3-methyl-substituted fatty acids, but also 2-methyl-substituted fatty acids and straight chain fatty acids.

In agreement with earlier data (18), the formation of CO_2 was clearly dependent on the presence of Fe^{3+} ions, as omission of Fe^{3+} from the incubation mixtures resulted in an almost complete suppression of CO_2 production. The stimulation of α-oxidation by phosphate and NADPH varied according to the substrate used. The reason for this variation is presently not known.

Addition of CoA to the incubation mixtures, fortified with Fe^{3+}, NADPH and phosphate, did not affect the decarboxylation rates. When CoA was added together with ATP, CO_2 production decreased, suggesting that fatty acid activation shunted the fatty acids away from the α-oxidation pathway. Why the decarboxylation of 3-methyl substituted fatty acids was affected more than that of palmitate and its 2-methyl derivative is not clear. In order to exclude the possibility that the CO_2 production from palmitate and 2-methylpalmitate, which can be degraded *via* β-oxidation, was derived from β-oxidation by contaminating mitochondria and/or peroxisomes, CO_2 production was measured in the presence of the cofactors required for β-oxidation. Under these conditions, hardly any CO_2 production could be detected.

Reverse-phase HPLC separation of α-oxidation products and intermediates. In an attempt to identify the reaction products and putative reaction intermediates of α-oxidation, we chose 3-methyl-[1-^{14}C,3-^{3}H]heptadecanoic and [1-^{14}C, 9,10-^{3}H]palmitic acids as the substrates for further experiments. The reverse-phase HPLC chromatograms of the ethyl acetate extracts from incubations of rat liver microsomes with double-labelled substrates are shown in Figure 1 (A and B). In each incubation, one single-labelled tritiated peak (P and P′) and several double-labelled peaks (A, B, C and I, II, III) were detected. In addition, we also detected a single ^{14}C-labelled peak, designated as D in Figure 1A. These peaks were all dependent on the presence of the cofactors and of protein and were not observed at zero time or upon incubation with boiled microsomes. The results suggest that the single-labelled tritiated peaks represent the decarboxylated α-oxidation products and that the double-labelled peaks may represent reaction intermediates. The peaks designated S and S′are the

TABLE 1

Cofactor Requirements and Substrate Specificity of α-Oxidation[a]

	Complete system[b]	$-Fe^{3+}$	$-P_i$[c]	$-NADP^+$[d]	+CoA[e]	+CoA + ATP[f]	β-Oxidation[g]
3-Methylheptadecanoate	89.4	0	0	2.52	92.1	5.05	0
Phytanate	73.2	1.26	8.65	4.68	72.3	5.95	0.36
Palmitate	100.7	0.54	39.3	18.6	107.8	52.6	1.80
2-Methylpalmitate	91.4	0	23.6	14.4	93.7	37.3	0.18

[a]The results are all expressed as pmoles of CO_2 produced per mg protein per min.
[b]The complete reaction mixture (final volume, 1 mL) consisted of 200 mM Tris buffer, pH 7.4; 0.1 mM $FeCl_3$; 4.0 mM phosphate buffer; 1.2 mM NADP; 5.0 mM glucose-6-phosphate; 2 units of glucose-6-phosphate dehydrogenase; 0.2 mM [1-^{14}C]fatty acid; and 0.5 mg microsomal protein. Incubations were carried out at 37°C for 15 min.
[c]In incubations listed in this column, the NADPH generating system was replaced by NADPH.
[d]$NADP^+$ was omitted from the complete system.
[e]CoA (0.5 mM) was added to the complete system.
[f]CoA (0.5 mM) and 4 mM ATP were added to the complete system.
[g]The incubation media contained in addition to the Tris buffer only the β-oxidation cofactors: 0.5 mM CoA, 0.5 mM carnitine, 4 mM ATP, and 2 mM NAD^+.

unreacted substrates. The peaks labelled P_{450} and P'_{450} probably correspond to ω-oxidation products as they almost completely disappeared when the incubations contained cytochrome C, which competes with cytochrome P_{450} for electrons from NADPH cytochrome P_{450} reductase, or an inhibitor of cytochrome P_{450}, such as metyrapone.

Identification of the decarboxylation products. The single-labelled tritiated fractions P and P'of Figure 1 (A and B) were further purified by reverse-phase HPLC after methylation with ethereal diazomethane. In a subsequent step the tritiated methyl esters were subjected to GC and combined GC/MS. Only one major peak, whose elution time corresponded to that of methylated 2-methylhexadecanoic and pentadecanoic acid, was observed in the GC profiles (data not shown). Also, the EI mass spectra of these fractions corresponded to those of 2-methylhexadecanoic and pentadecanoic acid methyl ester. Intense ions were associated with the typical McLafferty rearrangement fragments of 2-methyl fatty acid (*m/z* 88) and of straight chain fatty acid (*m/z* 74) methyl esters. Other ions that were identified belonged to the carboxymethoxy series, starting at *m/z* 101 (115, 143, 157, 185, 213, 227 and 241) for methylated 2-methylhexadecanoic acid and at *m/z* 87 (101, 129, 143, 157, 171, 199 and 213) for methylated pentadecanoic acid. The signals at *m/z* 227 (M – 57) and at 213 (M – 43) reflected the formation of another typical fragment of the methyl esters of 2-methyl fatty acids and straight chain fatty acids, respectively (data not shown).

Identification of 5-oxo-, 4-oxo-, 5-hydroxy- and 3-hydroxy-3-methylheptadecanoic acids. The lipids present in the fractions corresponding to peaks A, B, C, and D of Figure 1A were methylated with ethereal diazomethane and further purified by reverse-phase HPLC (data not shown). Subsequent treatment with BSTFA–TMCS did not affect the polarities of the methyl ester of fractions A, B and D but substantially reduced the polarity of the methyl ester of C as revealed by TLC analysis (silica gel 60, hexane/diethyl ether/acetic acid, 70:30:1, by vol); data not shown, indicating that peak C represented a hydroxylated fatty acid. Further analysis by GC of the BSTFA–TMCS-treated purified methyl esters showed in each instance only one major peak, the EI mass spectra of which are given in Figure 2.

As illustrated in Figure 2A, peak A of Figure 1A was identified as being due to 5-oxo-3-methylheptadecanoic acid (expected molecular mass of the methyl ester at *m/z* 312). The base peak located at *m/z* 158 is formed by cleavage between carbons 6 and 7 and transfer of a proton from carbon 7 to the 5-oxo group by a typical McLafferty rearrangement. Ions at *m/z* 143 and 197 represent the two ions formed by an α-cleavage to the 5-oxo group. Other ions identified include *m/z* 126 (158 – CH_3OH), 171 (157 + 14), 101 and 239 (the two cleavage ions α to the 3-methyl group), 253 (M^+ – $COOCH_3$) and 281 (M^+ – OCH_3).

Based on the fragmentation pattern in Figure 2B, peak B was identified as 4-oxo-3-methylheptadecanoic acid. Identified ions of the methyl ester include *m/z* 144 (McLafferty ion after cleavage β to the 4-oxo group), *m/z* 129 and 211 (cleavage ions α to the 4-oxo group), *m/z* 112 (144 –

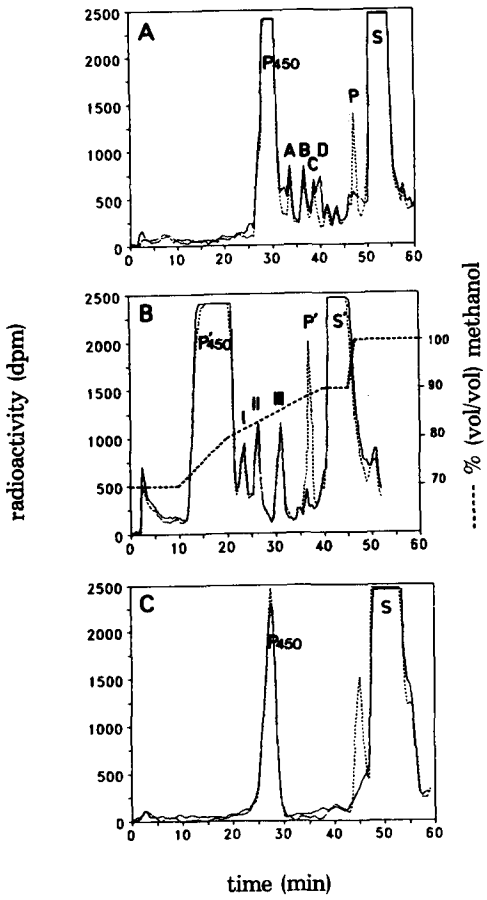

FIG. 1. Reverse-phase high-performance liquid chromatography (HPLC) separation of α-oxidation products and putative intermediates formed by liver microsomes and in rat hepatocytes. Rat liver microsomes (1 mg of protein) were incubated in capped siliconized vials at 37°C for 15 min in a mixture (final volume of 2 mL) consisting of 200 mM Tris buffer (pH 7.4), 12.5 mM sucrose, 0.25 mM 3-[*N*-morpholino]propanesulfonic acid, (pH 7.4), 0.1 mM $FeCl_3$, 1.2 mM NADP, 5 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 0.25 mM double-labelled fatty acid substrates. Reactions were terminated by the addition of 1 M HCl, and media were extracted with ethyl acetate as described in the Materials and Methods section. Isolated hepatocytes (5×10^6 cells) were incubated at 37°C in 2 mL Krebs-Henseleit buffer, pH 7.4, containing 2% (wt/vol) defatted albumin and 0.25 mM double-labelled 3-methylheptadecanoic acid. Reactions were stopped with $HClO_4$, and the fatty acid and its polar metabolites were extracted with chloroform/methanol and separated as described in the Materials and Methods section. The fatty acid substrates and the reaction products and intermediates were separated by reverse-phase HPLC using a methanol gradient in water containing 0.1% (vol/vol) acetic acid as shown in part B. The eluates were monitored for radioactivity (solid line, 14C; dashed line, 3H). A, Microsomes incubated with 3-methylheptadecanoic acid (3-methyl-[1-^{14}C]heptadecanoic acid; specific radioactivity, 1.5 μCi/μmol; 3-methyl-[3-^{3}H]heptadecanoic acid; specific radioactivity, 1.5 μCi/μmol). B, Microsomes incubated with palmitic acid ([1-^{14}C]palmitic acid; specific radioactivity, 1.5 μCi/μmol; [9,10-^{3}H]palmitic acid; specific radioactivity, 1.5 μCi/μmol) C, Hepatocytes incubated with 3-methylheptadecanoic acid (3-methyl-[1-^{14}C]heptadecanoic acid; specific radioactivity, 1.5 μCi/μmol; 3-methyl-[3-^{3}H]heptadecanoic acid, specific radioactivity, 1.5 μCi/μmol).

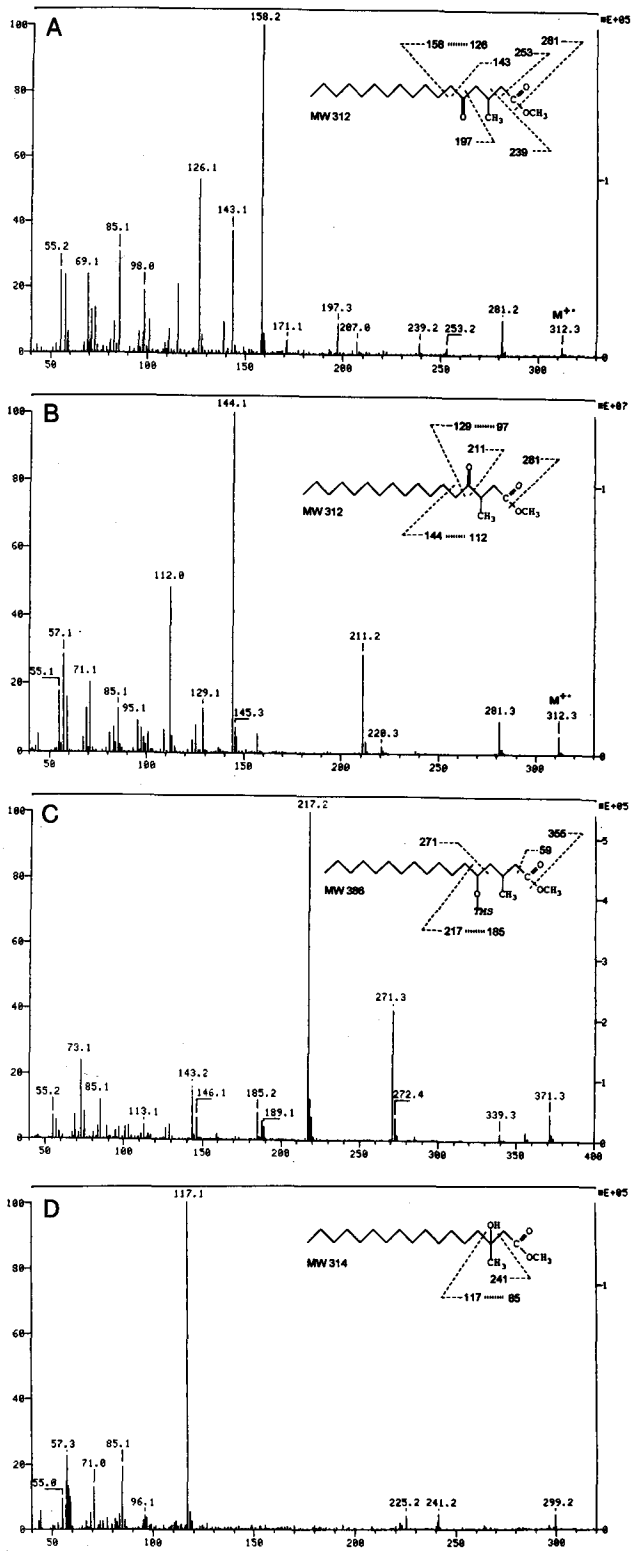

FIG. 2. Electron impact (EI) mass spectra of fractions A, B, C and D shown in Figure 1A after treatment with diazomethane and *bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilone (BSTFA–TMCS). Fractions A, B, C and D shown in Figure 1A were collected, methylated and further resolved by reverse-phase high-performance liquid chromatography, followed by gas chromatography/mass spectrometry after BSTFA–TMCS treatment of the purified methyl esters. EI mass spectra of peaks A (A), B (B), C (C) and D (D) are shown. MW, molecular weight.

CH_3OH), *m/z* 157 (143 + 14), 101 and 239, 281 and 312 (M^+).

As noted above, the differential mobility of the methyl ester of fraction C during TLC upon treatment with BSTFA–TMCS indicated that it was a hydroxylated fatty acid derivative. Based upon the EI spectrum, peak C was identified as 5-hydroxy-3-methylheptadecanoic. The highest ion mass is observed at *m/z* 371, which is due to a loss of a CH_3 group from a molecular ion of 386 (M^+), the methyl ester/trimethyl silyl (TMS) ether of a monohydroxylated 3-methylheptadecanoic acid. The base ion at *m/z*

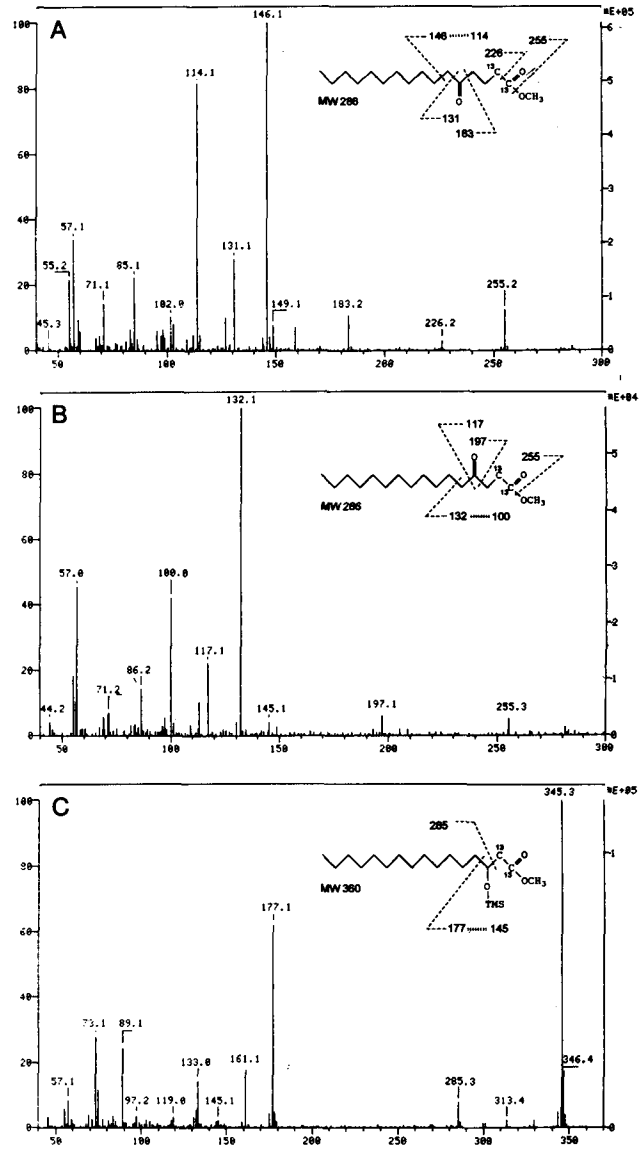

FIG. 3. EI mass spectra of fractions I, II and III shown in Figure 1B after treatment with diazomethane and BSTFA–TMCS. Fractions I, II and III shown in Figure 1B were collected, methylated and further resolved by reverse-phase high-performance liquid chromatography, followed by gas chromatography/mass spectrometry after BSTFA–TMCS treatment of the purified methyl esters. EI mass spectra of peaks I (A), II (B) and III (C) are shown. Abbreviations as in Figures 1 and 2.

217 is formed by a cleavage α to the OTMS group at the carbon 5 position, the other α-cleavage ion being observed at *m/z* 271. Other ions identified are at *m/z* 73 (TMS), 185 (217 – CH_3OH), 285 (271 + 14), 339 (371 – CH_3OH) and 355 (M^+ – OCH3).

Peak D in Figure 1A is only ^{14}C-labelled, suggesting that some chemical modification occurred at the carbon-3 position resulting in the loss of the ^3H label. TLC analysis indicated that the compound could not be silylated by treatment with BSTFA–TMCS after methylation with diazomethane (see above). The highest ion mass is observed at *m/z* 299 (Fig. 2D), which is in agreement with the molecular mass of a monohydroxylated 3-methylheptadecanoic acid methyl ester after loss of a methyl group (314 – 15). Therefore, this compound is easily identified as 3-hydroxy-3-methylheptadecanoic acid methyl ester. Such a tertiary alcohol cannot be silylated by treatment with BSTFA–TMCS due to steric hindrance. The base ion at *m/z* 117 and the ion at *m/z* 241 represent the two ions formed by cleavage α to the methyl and hydroxyl groups. Other ions identified are *m/z* 85 (117 – CH_3OH) and 225 (241 – 17 + 1).

Identification of 5-oxo-, 4-oxo- and 3-hydroxyhexadecanoic acids. The lipids associated with peaks I to III (Fig. 1B) were methylated with diazomethane and further purified by reverse-phase HPLC. GC analysis of these putative reaction intermediates derived from palmitic acid sometimes revealed more than one peak (data not shown). In order to facilitate the identification of the correct compound by MS, we decided in this particular experiment to incubate the microsomes with double-labelled palmitic acid diluted with [1,2-^{13}C]palmitic acid instead of the unlabelled palmitic acid. Figure 3 shows the EI mass spectra of the peaks corresponding to fractions I, II and III of Figure 1B, after treatment with diazomethane and BSTFA–TMCS. Preliminary TLC analysis indicated that fractions I and II did not contain a hydroxyl group, whereas fraction III appeared to be a hydroxylated fatty acid (data not shown).

Fraction I appeared to be 5-oxopalmitic acid (expected molecular ion at *m/z* 286) (Fig. 3A). The base ion is observed at *m/z* 146 (145 + 1, McLafferty ion after cleavage β to the 5-oxo group). The α-cleavage ions appear at *m/z* 131 and 183. Other ions identified include *m/z* 114 (146 – CH_3OH), 159 (145 + 14), 226 (M^+ – $^{13}COOCH_3$) and 255 (M^+ – OCH_3).

Based upon the fragmentation pattern, fraction II was identified as 4-oxopalmitic acid (Fig. 3B). The base ion at *m/z* 132 is a McLafferty ion after cleavage β to the 4 oxo group. Other ions identified include *m/z* 100 (132 – CH_3OH), 145 (131 + 14), and 255 (M^+ – OCH_3). Ions at *m/z* 117 and 197 represent the two ions formed by a cleavage α to the 4-oxo group.

The EI spectrum of the methyl ester/TMS ether of fraction III (Fig. 1B), identified as 3-hydroxypalmitic acid, is shown in Figure 3C. The expected molecular mass at *m/z* 360 is missing, which is not uncommon with the TMS derivatives of hydroxy fatty acids. The highest ion mass is observed at *m/z* 345 (360 – 15, a loss of methyl group). The hydroxyl group is identified at carbon-3, with one α-cleavage ion at *m/z* 177 and the other at *m/z* 285. Other ions identified include *m/z* 73 (TMS), 89 (OTMS), 145 (177 – CH_3OH), 313 (345 – CH_3OH) and 329 (M^+ – 31).

TABLE 2

Effects of Glutathione and Glutathione Peroxidase on Fatty Acid Decarboxylation[a]

	(pmole CO_2/min·mg·protein)	
	Palmitic acid	3-Methylheptadecanoic acid
Control	65.78	95.27
+ GSH (1 mM)	51.44	58.79
+ GPx (5 units)	73.40	88.55
+ GSH/GPx (1 mM/5 units)	5.30	6.61

[a]Incubations were carried out in the complete incubation mixture described in Table 1. GSH, glutathione; GPx, glutathione peroxidase.

Effects of glutathione and glutathione peroxidase on fatty acid decarboxylation by rat liver microsomes. The results of these experiments suggested that the compounds identified by GC/MS are not direct reaction intermediates. One possibility that we envision was that they might have been formed as rearrangement products of an unstable hydroperoxy fatty acid intermediate.

Therefore, in a subsequent experiment we examined the effects of glutathione and glutathione peroxidase on the decarboxylation of palmitic and 3-methylheptadecanoic acids by liver microsomes. Table 2 shows that glutathione plus glutathione peroxidase almost completely inhibited the decarboxylation reaction, again suggesting that a hydroperoxy fatty acid intermediate is formed during the reaction. Reverse-phase HPLC analysis of the ethyl acetate extracts revealed the disappearance of the tritiated decarboxylation products as well as of the double-labelled fractions A, B, C, I, II and III and the ^{14}C-labelled fraction D (Fig. 1). However, we did not detect the appearance of a hydroxy fatty acid, expected to be formed from the putative hydroperoxy fatty acid by glutathione peroxidase (data not shown).

Experiments with isolated hepatocytes. The results from these experiments did not allow for the identification of reaction intermediates. We therefore decided to carry out experiments with freshly isolated intact hepatocytes as enzyme source and 3-methylheptadecanoic acid as the substrate. The extracts from the incubations with hepatocytes were separated by reverse-phase HPLC, and the results are shown in Figure 1C. The single-labelled tritiated fraction was methylated, further purified by reverse-phase HPLC, and identified by GC/MS as the decarboxylation product 2-methylhexadecanoic acid (data not shown). The peak, designated S, represents unreacted 3-methylheptadecanoic acid. Only one other double-labelled peak, designated P_{450}, was observed. This peak completely disappeared after preincubation of the cells with metyrapone, indicating that the peak represents ω-oxidation products formed by cytochrome P_{450}.

DISCUSSION

This study demonstrates that, in the presence of the necessary cofactors, rat liver microsomes are capable of de-

carboxylating not only 3-methyl-branched fatty acids but also 2-methyl-branched fatty acids and even straight chain fatty acids. This is not surprising, as it has been shown that plant leaves as well as mammalian brain microsomes can catalyze the decarboxylation of straight chain fatty acids (21–23).

The main purpose of this study was the identification of the reaction products and putative reaction intermediates of α-oxidation. Tsai *et al.* (6,7) identified 2-hydroxyphytanic acid as a reaction intermediate in the α-oxidation of phytanic acid to pristanic acid. Ten Brink *et al.* (24) observed the accumulation of 2-hydroxyphytanic acid in plasma from patients with peroxisomal disorders, suggesting that in these patients, who also accumulate phytanic acid, the main defect lies in the conversion of 2-hydroxyphytanic acid to pristanic acid. However, Skjeldal and Stokke (25) have questioned the formation of a 2-hydroxy intermediate in α-oxidation.

In the present work, we have identified the decarboxylation products of palmitic and 3-methylheptadecanoic acids as pentadecanoic and 2-methylhexadecanoic acids, respectively, but we failed to find any evidence for the formation of a 2-hydroxy-fatty acid intermediate in our microsomal assay systems as well as in intact hepatocytes. Instead, in our microsomal system we identified the formation of 5-oxo-, 4-oxo-, 5-hydroxy- and 3-hydroxy-fatty acids. Obviously, these compounds are difficult to integrate in a mechanistic scheme of α-oxidation, producing a fatty acid one carbon shorter than the original molecule. Although their molecular structures seem to rule out a role as intermediates, their formation appears to be closely related to the decarboxylation process as studied under our assay conditions. Although their molecular structures seem to rule out a role as intermediates, their formation appears to be closely related to the decarboxylation process under our assay conditions. Like the production of CO_2 and of fatty acids shortened by one carbon atom, the formation of these intermediates was dependent on the presence of Fe^{3+}, NADPH and P_i, and it was strongly suppressed by the addition of glutathione and glutathione peroxidase. The fact that glutathione and glutathione peroxidase strongly inhibited fatty acid decarboxylation suggests that a 2-hydroperoxy fatty acid was formed as an intermediate in the decarboxylation reaction. The formation of a 2-hydroperoxy intermediate has also been suggested by others for fatty acid α-oxidation in plant leaves and mammalian brain microsomes (21–23, 26,27). Why we have not been able to detect the accumulation of a hydroxy fatty acid, which is formed from a hydroperoxy fatty acid by glutathione peroxidase, after addition of this enzyme to microsomes, is unclear unless one assumes that the putative hydroxy fatty acid remains tightly bound to the enzyme(s) involved in α-oxidation thus keeping its concentration low. In any case, at least in the microsomal system, the putative hydroxy fatty acid does not seem to function as an intermediate, because fatty acid decarboxylation was almost completely suppressed by glutathione peroxidase. On the other hand, if a hydroperoxy fatty acid is formed as an intermediate, this unstable compound might be rearranged to some stable oxygenated products, as has been reported for other hydroperoxy fatty acids (28–30). Such chemical rearrangement reaction, which is facilitated by the presence of iron ions (28,29), might explain the observed accumulation of the oxo and hydroxy fatty acids. The possibility that these oxygenated fatty acids were formed by reactive oxygen species, such as H_2O_2, superoxide anions or hydroxyl radicals, seems remote as their accumulation was not affected by inclusion in the reaction mixtures of catalase (H_2O_2 scavenger), superoxide dismutase (superoxide anion scavenger) and mannitol or dimethyl sulfoxide (hydroxyl radical scavengers) (data not shown).

The fact that we did not find any evidence for the accumulation of intermediates in isolated hepatocytes, suggests that in the intact cell the concentrations of intermediates must be low and that, if a hydroperoxy intermediate is formed, it must be rapidly converted by the α-oxidation system before significant chemical rearrangement can occur.

Because we have been unable to detect similar intermediates in incubations with microsomes and intact hepatocytes, our results do not allow us to conclude that the microsomal fatty acid decarboxylation system, as described here, represents the true α-oxidation process as it occurs in the intact cell. Although the different steps in this process are still unclear, our data show that the hydrogen at carbon-3 carrying the methyl branch is not involved in these steps, excluding a 2,3-desaturation as a potential reaction step.

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Upregulation of Low Density Lipoprotein Receptor Activity by Tumor Necrosis Factor, a Process Independent of Tumor Necrosis Factor-Induced Lipid Synthesis and Secretion

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It has been shown that tumor necrosis factor (TNF) rapidly upregulates expression of the low density lipoprotein (LDL) receptors on Hep G2 cells and acutely stimulates hepatic lipid synthesis and secretion *in vivo*. It may thus be possible that TNF-induced expression of LDL receptors is secondary to a decrease in cellular cholesterol content caused by TNF-stimulated lipid secretion. In order to know whether TNF upregulates LDL receptors by depletion of the cellular cholesterol content, the present experiments were designed to study the temporal relationship between TNF-stimulated expression of LDL receptor activity and TNF-induced changes in lipid synthesis and secretion in an *in vitro* setting by using Hep G2 cells (a highly differentiated human hepatoma cell line) as a hepatocyte model. Hep G2 cells were incubated with TNF (usually 2.5 nmol/L) for certain periods, and LDL receptor activity was evaluated by measuring [^{125}I]LDL binding at 4°C; lipid synthesis and secretion were assayed by measuring [^{3}H]glycerol incorporation into triglycerides and phospholipids as well as [^{14}C]acetate incorporation into cholesterol. We found that a 30-h exposure of the cells to TNF was needed for the effect of TNF to be seen on lipid synthesis and secretion as measured by incorporation of [^{3}H]glycerol into triglycerides and phospholipids, whereas TNF rapidly (in several hours) upregulated LDL receptor activity. TNF stimulated triglyceride synthesis, but did not stimulate phospholipid synthesis. On the other hand, TNF stimulated phospholipid secretion, but did not stimulate triglyceride secretion. Exposure of the cells to TNF for 16 or 24 h neither decreased cholesterol synthesis nor stimulated cholesterol secretion as measured by [^{14}C]acetate incorporation into cholesterol. Upregulation of LDL receptor activity through inhibition of cellular cholesterol synthesis with compactin (a competitive inhibitor of the 3-hydroxyl-3-methylglutaryl-CoA reductase) was augmented by TNF, whereas downregulation of LDL receptor activity through stimulation of cellular cholesterol synthesis with mevalonolactone almost completely blocked the upregulatory effect of TNF. In conclusion, TNF-stimulated expression of LDL receptor activity is not secondary to a depletion of cellular cholesterol content through TNF-stimulated lipid secretion or inhibition of cholesterol synthesis.

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Cytokines are believed to mediate various pathophysiologic responses to endotoxin and to infectious agents, of which tumor necrosis factor (TNF) is one of the most important (1–5). TNF is a mediator essential for the expression of most biological activities of endotoxin. Endotoxemia and gram-negative bacterial infection cause profound changes in metabolism, including hyperlipidemia. Administration of cytokines, such as TNF and interleukin (IL) 1, into experimental animals can also lead to a hyperlipidemic response (6–10; for reviews, see Refs. 11,12). TNF-induced hyperlipidemia occurs mainly through an acute stimulation of hepatic lipogenesis with a subsequent release of very low density lipoproteins (VLDL) into the bloodstream (6–8). Earlier studies have shown that TNF directly stimulates hepatic lipogenesis in Hep G2 cells (13). We and others have previously reported that TNF rapidly upregulates expression of the low density lipoprotein (LDL) receptors on Hep G2 cells (14,15). Thus, TNF may upregulate LDL receptor expression by decreasing hepatic cholesterol content through stimulation of VLDL secretion or by other mechanisms. The present study was carried out to elucidate the temporal relationship between TNF-stimulated expression of LDL receptors and TNF-induced changes in hepatic lipid synthesis and secretion in Hep G2 cells.

MATERIALS AND METHODS

Materials. Recombinant human TNF was supplied by Boehringer Ingelheim (Vienna, Austria). [1(3)-^{3}H]Glycerol (2.8 Ci/mmol), [9,10(n)-^{3}H]oleic acid (5 Ci/mmol) and [1-^{14}C]acetic acid (sodium salt, 53.4 mCi/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom). Oleic acid, triolein, cholesterol, cholesteryl oleate, fatty acid-free bovine serum albumin (BSA), human serum albumin (HSA; fraction V) and mevalonolactone were purchased from Sigma (St. Louis, MO). Compactin was a kind gift from Dr. Akira Endo (Fermentation Research Laboratories, Sankyo Co. Ltd., Tokyo, Japan). Fetal calf serum was purchased from Flow Laboratories (Irvine, United Kingdom). RPMI-1640 with glutamine and 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) were purchased from Life Technologies Ltd. (Paisley, United Kingdom). Plastic Petri dishes (35-mm) and 80-cm^2 flasks were purchased from NUNCLON (Roskilde, Denmark). Thin-layer chromatography (TLC) silica gel 60 plates were purchased from E. Merck (Darmstadt, Germany). OptiPhas HiSafe II liquid scintillation cocktails were purchased from Wallac Oy (Turku, Finland).

Cell culture. The human hepatoma cell line Hep G2 was obtained from the American Tissue Type Culture Collection (Rockville, MD). The cells were cultured in 80-cm^2 flasks and seeded into 35-mm Petri dishes for the experiments as described previously (16,17).

Lipoprotein isolation and labeling. LDL was isolated

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Abbreviations: apo, Apolipoprotein; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HSA, human serum albumin; IL, interleukin; LDL, low density lipoproteins; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; TNF, tumor necrosis factor; VLDL, very low density lipoproteins.

from human plasma from normocholesterolemic subjects by sequentional preparative ultracentrifugation (18) as described earlier (16,17). A narrow density range (1.034–1.054 g/mL) was used in the experiments to minimize contamination of LDL with apolipoprotein E.

LDL was iodinated with ^{125}I by the monochloride method (19) as modified for lipoproteins (20). Unbound ^{125}I was removed by chromatography on a Sephadex G-25 column PD10 (Pharmacia, Uppsala, Sweden), followed by extensive dialysis against 0.15 M NaCl with 1 mM EDTA, pH 7.4. The specific activity of the LDL was between 150 and 400 cpm/ng LDL protein.

Cellular lipid biosynthesis. To measure triglyceride and phospholipid synthesis, we used the [^{3}H]glycerol incorporation technique under essentially the same experimental conditions as cited by Grunfeld *et al.* (13). In brief, the effect of TNF on lipid biosynthesis in Hep G2 cells was studied after the cells had reached subconfluency. The cells were then incubated in RPMI 1640 containing 0.5% HSA in the presence or absence of TNF as described in the legends to the figures (shown later). For the last 6 h, the medium was replaced with RPMI 1640 containing 0.5% HSA, 1 mmol/L oleic acid (bound to albumin) and 2.5 μCi/mL [^{3}H]glycerol with or without TNF. Oleic acid was included during the incubation to enhance the synthesis of triacylglycerols. Glycerol incorporation was stopped by removing the medium and washing the cells three times with cold phosphate-buffered saline (PBS). The cellular lipids and the lipids secreted into the medium were then extracted with chloroform/methanol (1:2, by vol) (21). The chloroform phase was dried under nitrogen, after which the lipids were separated by TLC as described earlier (22). The triglyceride band was recognized by exposure to iodine vapor, using triolein as an internal standard, whereas phospholipids did not migrate from the origin. The triglyceride and phospholipid bands were then cut from the plates and counted in 10 mL scintillation fluid in a beta counter (Packard 300 CD liquid scintillation spectrometer; Packard Instruments, Groningen, The Netherlands).

To measure cholesterol synthesis, we used [^{14}C]acetate as precursor. Hep G2 cells were used when the cells had reached subconfluency. The cells were then incubated in RPMI 1640 containing 0.5% HSA in the presence or absence of TNF (2.5 nmol/L). For the last 16 h, [^{14}C]acetate (sodium salt, 2 μCi/mL medium) was added. [^{14}C]Acetate incorporation was stopped by removing the medium and washing the cells three times wth cold PBS. The cellular lipids and the lipids secreted into the medium were then extracted and separated by TLC as described in the above paragraph. The cholesterol band was cut from the plates and counted in 10 mL scintillation fluid in the beta counter. Radioactivity associated with the cholesterol band was taken as index of the overall cholesterol biosynthetic activity.

LDL receptor activity assay. The LDL receptor activity assay was carried out at 4°C by measuring LDL binding as described earlier (14,22). In brief, after exposure of the cells to TNF (2.5 nmol/L), the cells were washed twice with 1 mL PBS containing 0.2% BSA. Ice-cold medium (1 mL) containing 0.5% HSA was added to each dish. The cells were then precooled for 20 min at 4°C. Next, [^{125}I]LDL (2 μg LDL protein/mL medium) was added to each dish for a 2-h incubation at 4°C. The cells were then washed once with 1 mL of cold PBS containing 0.2% BSA and twice with 1 mL PBS, and then scraped into 1 mL of 0.5 mol/L NaOH for cell protein assay and for measuring radioactivity in a gamma-counter (LKB model 1271 automatic gamma counter; Wallac, Turku, Finland).

Cholesterol esterification assay. The cells were incubated with RPMI 1640 containing 0.5% HSA in the presence or absence of TNF (2.5 nmol/L), with increasing amounts of LDL added, for 24 h. For the last 4 h, [^{3}H]oleic acid complexed with BSA (2 μCi/mL medium) was added to measure cholesterol esterification as described previously (22). The radioactivity recovered in the cholesteryl ester band was taken as a measure of the cellular cholesteryl ester synthetic activity.

Statistics. Data are presented as means ± SD. Experimental results were analyzed for their statistical significance by Student's *t*-test. The paired data were analyzed by Student's paired *t*-test, as indicated in the text. A P value of less than 0.05 was considered significant.

RESULTS

Effect of TNF on cellular triglyceride and phospholipid synthesis. Incubation of Hep G2 cells with TNF (2.5 nmol/L or 42.5 ng/mL) did not acutely stimulate lipid synthesis, but after 30-h exposure of the cells to TNF, [^{3}H]glycerol incorporation into triglycerides was increased by 15–27% (Fig. 1A). An optimal effect of TNF on triglyceride synthesis was seen after 78 h. The cells were then exposed to increasing amounts of TNF for 78 h. The data show that TNF stimulated triglyceride synthesis in a dose-dependent manner (Fig. 2A). Thus 1.56 nmol/L of TNF induced an increase in triglyceride synthesis by about 21%. At concentrations over 3.125 nmol/L, the level of increased triglyceride synthesis (by 35–48%, $P < 0.05$ or less) remained quite constant. By contrast, TNF did not seem to stimulate phospholipid synthesis (Figs. 1A and 2A).

Effect of TNF on cellular triglyceride and phospholipid secretion. TNF did not stimulate triglyceride secretion, but caused a time- and dose-dependent stimulation of secretion of phospholipids into the medium (Figs. 1B and 2B). During shorter incubation (up to 16 h) with TNF (2.5 nmol/L), there was no increase in the secretion of newly synthesized lipids. After 30 h of incubation, TNF-treated cells seemed to secrete more labeled phospholipids into the medium than did control cells (a 19–31% increase) (Fig. 1B). Longer incubations with TNF led to a progressive increase in phospholipid secretion into the medium (Fig. 1B). TNF stimulated [^{3}H]glycerol incorporation into phospholipids in a dose-dependent manner (Fig. 2B). Thus, 0.78 nmol/L of TNF cause a 42% increase ($P < 0.02$) in phospholipid secretion. At concentrations over 1.56 nmol/L, the increase in phospholipid secretion (by 67–84%, $P < 0.001$) remained quite constant. It should also be noted that only relatively small amounts of newly synthesized lipids were secreted (see legends to Figs. 1 and 2).

Effect of TNF on cholesterol synthesis and secretion. After 16 or 24 h of exposure of cells to TNF (2.5 nmol/L),

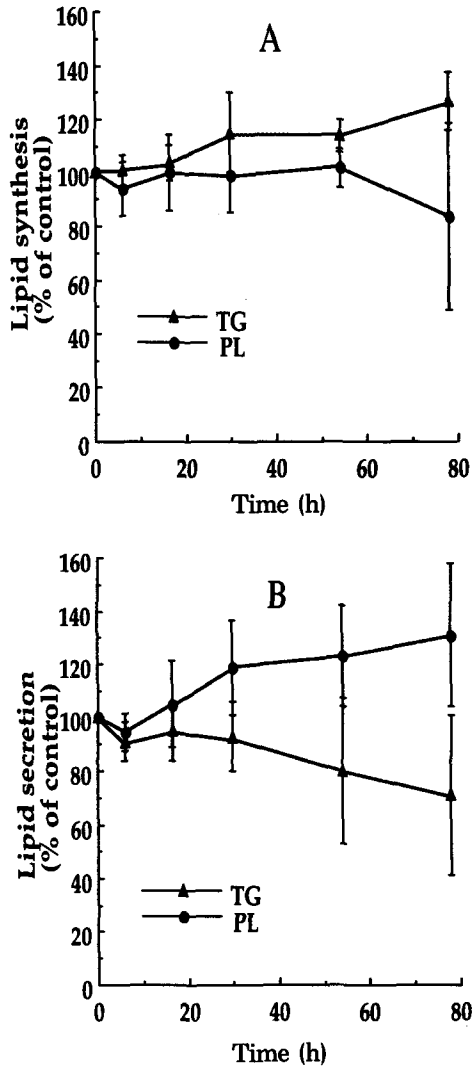

FIG. 1. Time course of the effect of tumor necrosis factor (TNF) on [^{3}H]glycerol incorporation into cellular lipids (synthesis) and into medium lipids (secretion). Hep G2 cells were cultured in 2 mL RPMI 1640 containing 10% fetal calf serum until subconfluent. Medium was then changed to 1 mL medium containing 0.5% human serum albumin (HSA) for 78 h. During this period of incubation, TNF was added at various time points at a concentration of 2.5 nmol/L. Total exposure time to TNF is indicated in the figure. For the last 6 h, medium was replaced with 1 mL medium containing 0.5% HSA, 1 mmol/L oleic acid (bound to albumin) and 2.5 μCi [^{3}H]glycerol with or without TNF. The cellular lipids and the secreted lipids were extracted and analyzed as described in the Materials and Methods section. The data are means ± SD of three separate experiments, each done at least with triplicate dishes and are expressed as percentages of controls. The control values for [^{3}H]glycerol incorporation into the cellular triglycerides (TG) and phospholipids (PL) (lipid synthesis) are 202.7 and 31.5 pmol/dish, respectively. The control values for [^{3}H]glycerol incorporation into the medium TGs and PLs (lipid secretion) are 1.1 and 1.3 pmol/dish, respectively.

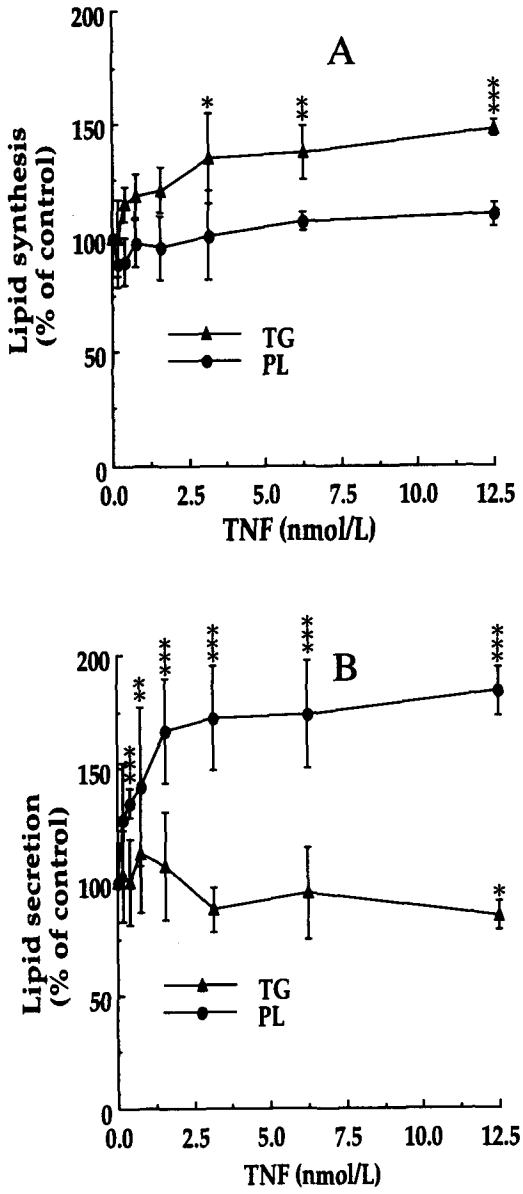

FIG. 2. Dose response of the effect of TNF on [^{3}H]glycerol incorporation into cellular lipids (synthesis) and into medium lipids (secretion). Hep G2 cells were cultured in 2 mL RPMI 1640 containing 10% fetal calf serum until subconfluent. Medium was then changed to 1 mL medium containing 0.5% HSA with increasing amounts of TNF for 78 h. For the last 6 h of the incubation, medium was replaced with 1 mL medium containing 0.5% HSA, 1 mmol/L oleic acid (bound to albumin) and 2.5 μCi [^{3}H]glycerol with increasing amounts of TNF. The cellular lipids and the secreted lipids were extracted and analyzed as described in the Materials and Methods section. The data are means ± SD of triplicate determinations and are expressed as percentages of controls. The control values for [^{3}H]glycerol incorporation into the cellular TGs and PLs (lipid synthesis) are 160.5 and 25.5 pmol/dish, respectively. The control values for [^{3}H]glycerol incorporation into the medium TGs and PLs (lipid secretion) are 0.9 and 1.3 pmol/dish, respectively. One, two and three star(s) show that *P* values are less than 0.05, 0.02 and 0.001, respectively. Abbreviations as in Figure 1.

no significant changes could be seen in [^{14}C]acetate incorporation into cellular cholesterol (98 ± 10% of the control for a 16-h exposure in three separate experiments each done with triplicate dishes; 98 ± 12% of the control for a

24-h exposure in four separate experiments each done with triplicate dishes). The data indicate that TNF does not acutely inhibit cellular cholesterol synthesis. However, a 48-h exposure to TNF seemed to slightly inhibit cellular cholesterol synthesis (85 ± 8.2% of the control in three separate experiments each done with triplicate dishes). On the other hand, after exposure to TNF for 16, 24, or 48 h the cells did not secrete more ^{14}C-labeled cholesterol into the medium (data not shown).

Comparison of TNF-stimulated lipid synthesis and secretion with its effect on expression of LDL receptors. Exposure of the cells to TNF caused a time-dependent increase in LDL receptor activity in Hep G2 cells. After 16 h of incubation, TNF-stimulated LDL binding seemed to reach a maximal level (14). Because TNF does not increase nonspecific binding of [^{125}I]LDL to Hep G2 cells (determined in the presence of a 40-fold excess of unlabeled LDL), the increase in binding exclusively reflects increased specific binding (calculated by subtracting nonspecific binding from total binding) i.e., increased LDL receptor activity (14). Consequently, in the present study only total binding was measured. The results from eight separate experiments (including three matched experiments in which LDL receptor activity and lipid synthesis and secretion were measured simultaneously) are shown in Table 1. After a 16-h exposure of the cells to TNF (2.5 nmol/L), LDL binding increased by 49% ($P < 0.005$, Student's paired t-test), whereas no significant changes could be seen in lipid synthesis and secretion (see Table 1). In fact, TNF-stimulated LDL receptor activity became apparent earlier (after a 2-h exposure of the cells to 2.5 nmol/L TNF, an increase of 16 ± 9.9%, $P < 0.025$, Student's paired t-test, five separate experiments; after a 12-h exposure of the cells to 2.5 nmol/L TNF, an increase of 43 ± 15%, $P < 0.001$, Student's paired t-test, eighteen separate experiments). After longer exposure to TNF (48 h), TNF increased LDL binding by 62% ($P < 0.05$, Student's paired t-test), increased triglyceride synthesis by 15% ($P < 0.005$, Student's paired t-test), and increased phospholipid secretion by 27% ($P < 0.025$, Student's paired t-test), respectively (see Table 1).

Effect of TNF on LDL-stimulated cholesterol esterification. One of the consequences of LDL receptor-mediated cellular LDL catabolism is the activation of acyl-coenzyme A:cholesterol acyltransferase. LDL-induced cholesterol esterification reflects LDL receptor activity. Exposure of Hep G2 cells to TNF (2.5 nmol/L) caused a significant increase in LDL-stimulated cholesterol esterification, but had no effect on basal cholesterol esterification (in the absence of LDL) (Table 2). This implies that TNF-stimulated LDL receptor activity leads to an increase in cellular endocytosis of LDL followed by hydrolysis of cholesteryl ester into free cholesterol in lysosomes.

Modulation of cellular cholesterol content and its effect on TNF-induced upregulation of LDL receptor activity. We used compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, to inhibit cholesterol synthesis. We used mevalonolactone, which enhances cellular cholesterol synthesis by supplying an intermediary in the cholesterol biosynthetic pathway beyond the rate-limiting step, to stimulate cholesterol synthesis. We then observed the effect of variation in cellular cholesterol content on TNF-stimulated expression of LDL receptor activity. As expected, exposure of the cells to mevalonolactone for 48 h led to a marked reduction of LDL binding by 69–75% ($P < 0.001$), whereas exposure to compactin caused an increase in LDL binding by 156–173% ($P < 0.001$) (Table 3). Moreover, upregulation of LDL receptor activity with compactin showed a synergistic effect with the upregulation by TNF, whereas downregulation of LDL receptor activity with mevalonolactone almost completely blocked the upregulatory effect of TNF (Table 3).

DISCUSSION

TNF is a mediator responsible for most *in vivo* endotoxic activity (1–5). TNF may mediate endotoxin-induced hyperlipidemia by suppression of lipoprotein lipase (23,24), an enzyme which hydrolyzes triglycerides in VLDL and chy-

TABLE 1

Comparison of the Effect of TNF on LDL Receptor Activity with Its Effect on Synthesis and Secretion of Lipids[a]

Exposure time to TNF	LDL receptor activity		Lipid synthesis				Lipid secretion			
			TG		PL		TG		PL	
	16 h	48 h	16 h	48 h	16 h	48 h	16 h	48 h	16 h	48 h
Experiment number										
1	126 ± 18	132 ± 19[b]	100 ± 7.9	117 ± 0.8[b]	102 ± 10	110 ± 3.7	92 ± 3.2	97 ± 3.5	109 ± 7.4	137 ± 5.9[c]
2	136 ± 3.3[d]	171 ± 30[c]	104 ± 4.4	118 ± 5.2	102 ± 2.6	112 ± 2.7[c]	95 ± 5.8	82 ± 6.2[c]	110 ± 11	131 ± 8.8[c]
3	149 ± 13[f]	203 ± 16[f]	105 ± 1.2	112 ± 9.6	110 ± 3.1	107 ± 3.9	94 ± 17	83 ± 3.4[c]	91 ± 3	110 ± 1.5
4	—	—	97 ± 0.3	109 ± 4	90 ± 7.7	97 ± 3.3	85 ± 14	51 ± 8.4[e]	123 ± 38	114 ± 27
5	—	—	110 ± 6.7	121 ± 10[b]	—	—	107 ± 17	105 ± 24	102 ± 45	145 ± 25[c]
6	183 ± 15[e]	142 ± 14[c]	—	—	—	—	—	—	—	—
7	159 ± 12[e]	—	—	—	—	—	—	—	—	—
8	138 ± 6.8[f]	—	—	—	—	—	—	—	—	—
Sum (mean ± SD)	149 ± 20[e,g]	162 ± 32[b,g]	103 ± 5	115 ± 5[e,g]	101 ± 8	106 ± 6	95 ± 8	84 ± 21	107 ± 12	127 ± 15[c,g]

[a]Subconfluent cells were used. After exposure of the cells to tumor necrosis factor (TNF) (2.5 nmol/L) for 16 or 48 h, low density lipoprotein (LDL) binding and [^{3}H]glycerol incorporation into triglycerides and phospholipids were measured as described in the Materials and Methods section. The data are means ± SD of, at least, triplicate determinations, and expressed as percentages of the controls. TG, triglycerides; PL, phospholipids. [b]$P < 0.05$. [c]$P < 0.025$. [d]$P < 0.01$. [e]$P < 0.005$. [f]$P < 0.001$. [g]Student's paired t-test.

TABLE 2

Effect of TNF on LDL-Induced Cholesterol Esterification[a]

LDL added (μg LDL protein/mL medium)	[^{3}H]Oleate incorporation into cholesteryl ester (fmol/dish)			P value, compared with the corresponding control values
	Control (without TNF)	TNF		
0	682 ± 75	660 ± 81	(97)	NS
12.5	1248 ± 177	1824 ± 56	(146)	<0.01
25	1785 ± 156	2582 ± 450	(144)	<0.05
50	1768 ± 175	3047 ± 243	(172)	<0.005
100	2065 ± 91	2905 ± 240	(141)	<0.005

[a]Hep G2 cells were used when the cells reached subconfluency. The cells were incubated with RPMI 1640 containing 0.5% human serum albumin in the presence or absence of TNF (2.5 nmol/L) with increasing amounts of LDL added for 24 h. For the last 4 h, [^{3}H]oleic acid complexed with bovine serum albumin was added for the cholesterol esterification assay as described in the Materials and Methods section. The data are means ± SD of triplicate determinations. Numbers in parentheses are percentages of the corresponding control values. NS, not significant. Abbreviations as in Table 1.

TABLE 3

Effect of Variation of Cellular Cholesterol Content on TNF-Induced Upregulation of LDL Receptor Activity[a]

Additives	LDL binding (% of control)	
	Experiment 1	Experiment 2
None (control)	100 ± 13	100 ± 13
Mevalonolactone (10 mM)	25 ± 3.3[b]	31 ± 3.8[b]
Compactin (20 μM)	273 ± 22[b]	256 ± 24[b]
TNF (2.5 nM)	195 ± 17[c]	177 ± 26[d]
Mevalonolactone (10 mM) + TNF (2.5 nM)	29 ± 4.2[b]	33 ± 2.3[b]
Compactin (20 μM) + TNF (2.5 nM)	395 ± 59[c,e]	350 ± 31[b,f]

[a]Hep G2 cells were cultured in RPMI1640 containing 10% fetal calf serum until subconfluent. Medium was then changed to RPMI 1640 containing 0.5% human serum albumin with or without mevalonolactone or compactin for 48 h. For the last 24 h, TNF (2.5 nmol/L) was added to the dishes as indicated. After the incubation, LDL binding was measured as described in the Materials and Methods section. The data are means ± SD of triplicate determinations and expressed as percentages of the controls. The control values were 15.6 ng LDL protein/mg cell protein (experiment 1) and 15.5 ng LDL protein/mg cell protein (experiment 2), respectively. Abbreviations as in Table 1.
[b]$P < 0.001$, compared with the corresponding control values.
[c]$P < 0.005$, compared with the corresponding control values.
[d]$P < 0.02$, compared with the corresponding control values.
[e]$P < 0.05$, compared with the addition of compactin alone, and $P < 0.001$, compared with the addition of TNF alone.
[f]$P < 0.02$, compared with the addition of compactin alone, and $P < 0.005$, compared with the addition of TNF alone.

lomicrons, and/or by stimulation of hepatic lipid synthesis with a subsequent release of endogenous triglycerides as VLDL particles into the bloodstream (6–8). Current data show that TNF increases serum triglyceride levels primarily by stimulation of hepatic lipid synthesis and secretion of VLDL, rather than by suppression of lipoprotein lipase (6–9,12). Administration of TNF leads to an acute hypertriglyceridemic response, which can be seen as early as 2 h after TNF administration (6–8). We and others have previously reported that TNF rapidly upregulates LDL receptor activity *in vitro* (14,15). Thus TNF may upregulate LDL receptor expression *via* depletion of hepatocytic cholesterol content secondarily caused by stimulation of VLDL secretion. Therefore, we compared the effects of TNF on lipid synthesis and secretion and on expression of LDL receptor activity in Hep G2 cells.

Although the use of Hep G2 cells has some limitations, for example, these cells secrete only scarce amounts of lipoproteins which also applies, to some extent, to hepatocytes in primary cultures (25–27), Hep G2 cells have been used extensively as a model for studying hepatic lipid metabolism (28). Grunfeld *et al.* (13) showed that TNF and other cytokines (IL 1 and interferon α) stimulate lipid synthesis and secretion in Hep G2 cells by measuring [^{3}H]glycerol incorporation into lipids. They found that a 24-h exposure of Hep G2 cells to TNF is needed for TNF to stimulate lipid synthesis and secretion (13), although data were mostly given as incorporation of [^{3}H]glycerol into total lipids. By using the same experimental conditions, the present study confirms that TNF can directly stimulate lipid synthesis and secretion. The response patterns of Hep G2 cells to TNF exposure found in the present study are basically consistent with the data by Grunfeld *et al.* (13), i.e., TNF stimulates lipid synthesis and secretion in a dose- and time-dependent manner, but long-term exposure is needed for the effect to appear. We further found that TNF stimulated incorporation of [^{3}H]glycerol into triglycerides in Hep G2 cells, but did not stimulate phospholipid synthesis, and that TNF caused an increase in phospholipid secretion, but did not stimulate triglyceride secretion. This suggests that TNF may cause triglyceride accumulation in hepatocytes. One recent *in vitro* study showed that after exposure of Hep G2 cells to cytokines (TNF, IL 1 and IL 6) for 24 h, the concentrations of apolipoproteins (apo) A-I and B in the medium were decreased and cellular apoA-I mRNA, but not apoB mRNA, was decreased (29). In *in vivo* studies, it was found that the levels of hepatic mRNA for apoB and apoE did not increase 24 h after a single administration of TNF (30), but hepatic lipid (fatty acids, triglycerides and cholesterol) synthesis and secretion were markedly increased (6–8). *In vivo* stimulation of triglyceride synthesis by TNF is fast compared to its stimulation of cholesterol synthesis (6–8). Hence, TNF may relatively delay secretion of triglycerides *in vivo,* resulting in hepatic accumulation of triglycerides, a process involved in fatty liver formation, which is also observed in endotoxemia (31).

The lipogenetic response of Hep G2 cells to TNF in *in vitro* studies carried out by us and others (13) is significantly different from that which is seen in an *in vivo* setting. TNF administration to intact animals causes an acute response of lipid synthesis and secretion by the liver as early as 2 h after administration (6–8), whereas many hours are needed for the lipogenetic effect of TNF to appear in Hep G2 cells. What causes this difference is not clear. This is also the case with other biological activities of TNF, i.e., serum zinc levels and amino acid transport, which are rapidly modulated when TNF is given to rats

in vivo, but TNF increases zinc and amino acid transport only after 16 to 20 h of exposure in hepatocytes *in vitro* (32,33).

LDL receptor expression is believed to be largely controlled at the level of transcription and has been linked inversely to intracellular cholesterol levels (34,35), whereas little is known about the regulatory mechanisms for LDL receptor activity independent of cholesterol pathways. The current study shows that TNF acutely stimulates LDL receptor activity, whereas at this point, it neither increases lipid secretion nor decreases cholesterol synthesis. These findings suggest that TNF upregulates LDL receptor activity *via* a pathway independent of cholesterol homeostasis. One recent study has shown that TNF upregulates LDL receptors by inducing LDL receptor gene transcription in Hep G2 cells (15). Inhibition of protein synthesis blocks the stimulatory effect of TNF on expression of LDL receptor activity and LDL receptor gene (14,15), suggesting that TNF induces synthesis of protein factor(s) which then activate(s) LDL receptor gene transcription. It was found that TNF simultaneously induced expression of the genes for the LDL receptor and its positive transcription factor SP-1 in human endothelial cells (36), suggesting a possible role of SP-1 in TNF-induced expression of the LDL receptor. Other macrophage-derived factors, including oncostatin M, IL 6, IL1 and transforming growth factor β, have recently been reported to also have the ability to upregulate LDL receptor activity in Hep G2 cells (37,38).

We previously showed that incubation of Hep G2 cells with LDL for 12 h before exposure to TNF completely abolished the upregulatory effect of TNF on LDL receptor activity (14). In the present study, we further show that downregulation of LDL receptor activity *via* stimulation of cholesterol synthesis by using mevalonolactone also completely blocked the upregulatory effect of TNF on LDL receptor activity, whereas upregulation of LDL receptor activity *via* inhibition of cholesterol synthesis by using compactin showed a synergistic effect with TNF. One recent study also showed that LDL blocked the stimulatory effect of TNF on the expression of the LDL receptor gene (15).

The present study further shows that exposure of Hep G2 cells to TNF causes a significant increase in LDL-stimulated cholesterol esterification, suggesting that TNF-stimulated LDL receptor activity leads to an increase in cellular endocytosis of LDL followed by hydrolysis of cholesteryl ester into free cholesterol in lysosomes.

In summary, TNF-stimulated expression of LDL receptor activity is not secondary to a depletion of cellular cholesterol content caused by TNF-stimulated lipid secretion or by inhibition of cellular cholesterol synthesis, but rather an effect on LDL receptor gene expression. TNF-stimulated LDL receptor expression may be one example of non-cholesterol-mediated modes for the regulation of hepatic LDL receptors.

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Lipid Composition of Glucose-Stimulated Pancreatic Islets and Insulin-Secreting Tumor Cells

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The effect of glucose stimulation (25 mM for 5 min) on the phospholipid and neutral lipid composition of isolated pancreatic islets was studied to find out whether there is a change in the mass of potential lipid mediators or modulators of insulin secretion. For comparison, the lipid compositions of homogenates and subcellular fractions from RINm5F insulin-secreting tumor cells and of glucose-stimulated streptozotocin/nicotinamide-induced islet cell tumors were analyzed. After separation of the lipid extract into a neutral and an acidic fraction by anion-exchange chromatography, lipids were separated by high-performance thin-layer chromatography and quantitated by *in situ* densitometry of the cupric sulfate-charred bands. In glucose-stimulated islets, the molar percentages of phosphatidic acid (PA) and of phosphatidylinositol were significantly increased (3.1 vs. 4.7 mol% and 8.6 vs. 11.8 mol%), while those of all other phospholipids and neutral lipids, including 1,2-diacylglycerol, were not significantly changed. In stimulated islet cell tumors, an increase of PA was visible in the microsomal fraction, and there was an increase of lysophosphatidylcholine in the mitochondrial fraction. However, in both tumoral tissues, particularly in RINm5F cells, the lipid distribution pattern showed abnormalities which can be regarded as a loss of differentiation and which limit the usefulness of these tissues for the study of the physiological regulation of lipid metabolism during glucose stimulation. In conclusion, the data are in accordance with a role of PA early in stimulus-secretion coupling. The well-known stimulation of phospholipid synthesis in pancreatic islets during glucose-induced insulin secretion does not result in an increase in the total phospholipid mass.

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The role of pancreatic B cell lipid metabolism in the course of insulin secretion has remained elusive in spite of the considerable research efforts that have been made (for recent reviews, see Refs. 1 and 2). Stimulation of lipid metabolism during glucose-induced insulin secretion has been known for a number of years (3,4), and two, not mutually exclusive,views of its role have evolved: (i) lipid metabolism provides the pancreatic B cell with additional fuel resulting in a potentiation of glucose-induced insulin secretion (5–8); and (ii) lipid and, particularly, phospholipid metabolism generates second messengers which activate the secretory machinery (9–11). Following the discovery of phospholipase C-generated inositol-1,4,5-trisphosphate as Ca^{2+}-mobilizing second messenger (12) and of 1,2-diacylglycerol (1,2-DG) as an endogenous activator of protein kinase C (13), both of which are involved in stimulus-secretion coupling in a number of tissues, the second view also prevailed in studies on lipid metabolism in endocrine pancreatic tissue. However, there is a fundamental difference between stimulus-secretion coupling in pancreatic B cells and other secretory cells: The physiological induction of secretion in pancreatic islet B cells is strictly dependent on metabolism of the secretagogues ("substrate site" or "fuel" hypothesis of insulin secretion). It now appears that activation of phospholipase C is restricted to a cholinergic enhancement of glucose-induced insulin secretion or is only secondary to Ca^{2+} entry into the B cell during glucose-induced secretion (14). The physiological importance of lysophospholipid (15,16) and arachidonic acid liberation (17) due to phospholipase A_2 (PLA_2) activity in pancreatic islets (18) is still uncertain. The same applies to the recent description of a phospholipase D (PLD) activity (19), which results in the production of phosphatidic acid (PA).

Thus, in recent years a wealth of data has accumulated on the actions of putative lipid-derived second messengers and on their relative turnover rates, as measured by the incorporation of radioactively labeled precursors, but there is comparatively little information on the mass of phospholipids and neutral lipids in pancreatic islets and none on their subcellular distribution because comparatively large amounts of tissue are required. Some studies on this topic have been published (Refs. 3, 20–22; and see Table 1 for an overview), but only one of these has dealt with stimulated islets for a limited number of phospholipids (21). Mass measurements are particularly necessary to assess the role of lysophospholipids and of PA. Because both compounds are obligate intermediates in phospholipid metabolism, they are likely to become more intensively labeled during stimulation when determined by the incorporation of a radioactively labeled precursor. However, it is not clear whether an accumulation occurs that might enable these compounds to influence cellular functions, i.e., to exert a second messenger role. We therefore determined a detailed pattern of phospholipids and neutral lipids of resting and glucose-stimulated islets. As it is also desirable to study the subcellular distribution of the lipids, a similar pattern was determined for the homogenates and crude subcellular fractions of RINm5F cells and of a number of streptozotocin/nicotinamide (STZ/NA)-induced islet cell tumors to assess whether these tumoral tissues might be useful tools to study the

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Abbreviations: CHOL, cholesterol; CL, cardiolipin; DG, diacylglycerol; FA, unesterified fatty acids; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPTLC, high-performance thin-layer chromatography; L, prefix designating the 2-lysoform (e.g., LPC) of a phospholipid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PLD, phospholipase D; PS, phosphatidylserine; SPH, sphingomyelin, SPH1 denoting the low R_f and SPH2 the high R_f species; STZ/NA, streptozotocin/nicotinamide; TG, triacylglycerol.

TABLE 1

Comparison of Literature Data on Phospholipid Composition of Homogenates from Unstimulated Pancreatic Islets of Various Rodents[a]

Phospholipid	Ob/ob mice (Ref. 3)	NMRI mice (Ref. 20)	Sprague-Dawley rats (Ref. 21)	Wistar rats (Ref. 22)
LPC	n.d.	3.1	7.3	n.d.
SPH1 + SPH2	8	7.0	n.d.	10.7
PC	56	57.1	54.4	45.8
LPE	n.d.	n.d.	n.d.	n.d.
PE	11	23.7	23.8	20.6
PI	12	4.5	6.9	8.9
PS		4.4	6.4	14.0
PG	13	n.d.	n.d.	n.d.
PA		(<2)	n.d.	n.d.
CL	n.d.		1.2	n.d.
Σ (nmol/mg)	230	220	152	320

[a]The data are given as mol% for easier comparison between the different sets of data. The sums (Σ) have been recalculated on the basis of a protein content corresponding to half of the dry weight (Ref. 3) or an islet protein content of 0.5 μg (Refs. 20–22). In References 21 and 22, data on the neutral lipid content were also given, the sum being 4–6 pmol/islet [about 10 nmol/mg, excluding cholesterol (CHOL)] in Ref. 21, and 1043 ng/islet (about 3500 nmol/mg, including CHOL and cholesteryl ester) in Reference 22. PA mass in islet tissue was determined by Dunlop *et al.* (10) and can be recalculated to correspond to 65–130 nmol/mg protein. n.d., Not determined. LPC, lysophosphatidylcholine; SPH1, denotes the low R_f of sphingomyelin; SPH2, denotes the high R_f of sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin.

relation between stimulus-secretion coupling and lipid metabolism in pancreatic islets.

MATERIALS AND METHODS

Chemicals. Phospholipids and neutral lipids which were used as standards for chromatography were obtained from Sigma (St. Louis, MO), Calbiochem (San Diego, CA) or Serdary (London, Ontario, Canada). The purity was checked before use by high-performance thin-layer chromatography (HPTLC). Solvents were of high-performance liquid chromatography grade from Merck (Darmstadt, Germany), Baker (Deventer, The Netherlands) or Fluka (Buchs, Switzerland), as purissimum grade or as Burdick and Jackson brand. Sephadex media were obtained from Pharmacia (Uppsala, Sweden). HPTLC $60F_{254}$ plates were from Merck.

Tissue preparation and incubation. Pancreatic islets from fasted ob/ob mice (50–60 g) were isolated from the pancreas following collagenase digestion in Krebs-Ringer bicarbonate medium (23) and collected under a stereomicroscope. Due to the high yield of islets from the pancreas of these mice and the particularly large size of these islets, a tissue mass could be attained that was sufficient for a detailed analysis of phospholipids and neutral lipids of the homogenate, but was still insufficient for comparable analyses of subcellular fractions. An additional advantage of these islets is the high content (>90%) of B cells (24). After preincubation for 30 min in an *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Krebs-Ringer bicarbonate medium containing 5 mM glucose, batches of 750–1000 islets were incubated for 5 min in the same medium, containing either 5 or 25 mM glucose. After the incubation, the islets were homogenized in ice-cold homogenization medium [210 mM mannitol, 70 mM sucrose, 20 mM HEPES and 0.5 mM ethyleneglycol-*bis*(2-aminoethyl ether)*N,N,N',N'*-tetraacetic acid, adjusted to pH 7.0 with KOH], and the homogenate was extracted with hexane/2-propanol.

The RINm5F rat islet tumor cell line, which responds abnormally to glucose stimulation (25), was cultivated in Hams F12 medium supplemented with 10% fetal calf serum under an atmosphere of 95% O_2 and 5% CO_2 at 37°C. After trypsination, the cells (*ca.* 5×10^7) were washed in homogenization medium (see previous paragraph) and homogenized.

Pancreatic islet cell tumors with a high content of B cells (>90%) were induced in Wistar rats by STZ/NA treatment as described by STZ/NA treatment as described previously (26,27). After 15–18 mon, pancreatic islet tumors of variable size had developed in six rats. The pancreases were removed, and the tumors were isolated by microdissection under a stereomicroscope. After preincubation for 30 min at 5 mM glucose, three STZ/NA-induced tumors (wet weight 3.8, 14 and 71 mg) were stimulated with 25 mM glucose for 30 min, while the other three tumors (wet weight 6.8, 98 and 500 mg) served as controls. In view of the much larger tissue mass as compared to islets, this longer incubation time was chosen to ensure complete diffusion of glucose.

Subcellular fractionation. Crude subcellular fractions from RINm5F cells and islet cell tumors were obtained by differential centrifugation at 4°C. After centrifuging twice at $660 \times g$, the mitochondrial fraction was isolated from the supernatant by sedimentation at $4{,}000 \times g$ for 15 min. The pellet obtained after a $100{,}000 \times g$ centrifugation was the microsomal fraction, and the $100{,}000 \times g$ supernatant was the cytosolic fraction. By this centrifugation protocol, a fivefold enriched mitochondrial fraction was obtained, as only 10% of the B cell mitochondria were not pelleted by the $4{,}000 \times g$ step according to the determination of the mitochondrial marker enzymes, monoamine oxidase, succinic acid dehydrogenase and glutamic acid dehydrogenase (S. Lenzen, unpublished data). Secretory granules were mainly present in the microsomal fraction, but were also present in the mitochondrial fraction, as was visible from the insulin content (S. Lenzen, unpublished data). There is a close similarity between the subcellular composition of normal islets and STZ/NA tumors according to the determination of subcellular marker enzymes (27).

Lipid analysis. Lipids were extracted from the homogenates or the subcellular fractions by vortex-mixing and sonicating the samples with a twentyfold volume of hexane/2-propanol (3:2, by vol) (28). Centrifugation at $2{,}500 \times g$ pelleted the lipid-insoluble compounds. The supernatant was concentrated in a vacuum centrifuge (aRVC; Sigma-Zentrifugen, Osterode, Germany). This

total lipid extract was separated into a neutral and an acidic fraction by passage through a diethylaminoethyl-Sephadex minicolumn (1 mL gel volume, acetate as counter-ion). The neutral lipids and the zwitterionic phospholipids were eluted with 1.5 mL of Solvent A (chloroform/methanol/water, 30:60:8, by vol) (30), and the acidic phospholipids were eluted with 5 mL of Solvent B (chloroform/methanol/0.8 M sodium acetate, 30:60:8, by vol) (30). Both fractions were concentrated, redissolved in 300 µL of Solvent A and desalted by application onto a Sephadex G15 minicolumn (1.6 mL gel volume) and elution with 1.3 mL of Solvent A. The effluent of this column was concentrated again and aliquots applied to a HPTLC plate by use of a motorized Hamilton syringe. The neutral fraction (Fig. 1) was chromatographed with chloroform/methanol/triethylamine/water (30:35:34:8, by vol), while the acidic fraction (Fig. 2) was chromatographed with chloroform/methanol/triethylamine/2.6 M ammonia (30:37:34:8, by vol). The plates had previously been impregnated with boric acid from 40–100 mm for the development of the neutral fraction and from 10–100 mm for the development of the anionic fraction. The neutral lipids of the pancreatic islets (Fig. 3) were separated by chromatography of a desalted total lipid extract using a modification of a unidimensional multiple development system according to Yao and Rastetter (31) consisting of chlorobenzene/diethyl ether/ethanol/acetic acid (60:40:1:0.05, by vol), hexane/diethyl ether (94.6, by vol), and pure hexane. After development, the lipid bands were charred at 175°C with a cupric sulfate reagent (32). Quantification of the charred bands was performed in a Desaga CD60 densitometer by comparison with peak areas of standards run on the same plate. The precision of the standard curve, calculated as a polynomial of second degree, was of particular importance for the lower limit of determination of low abundance compounds, such as phosphatidylglycerol (PG), PA or 1,2-DG. Hexadecylphosphocholine, a synthetic phospholipid (synthesized and provided by Dr. H.J. Eibl, Max-Planck-Institute of Biophysical Chemistry, Göttingen), could be used as an internal standard due to its migration between LPC (L designates the 2-lysoform of a phospholipid; PC, phosphatidylcholine) and sphingomyelin (SPH) in HPTLC (33); its recovery was 89% in this system. Standard phospholipids, when processed through the different separation and concentration steps, suffered a loss of 10–15%. All determinations were made in duplicate. [For further details see Rustenbeck and Lenzen (33), Rustenbeck *et al.* (34), and references therein.] The mass of each class of lipid was determined as nmol per mg protein. To describe the phospholipid composition, the molar mass of an individual phospholipid class was expressed as percentage of the total phospholipid mass (mol%) of the respective

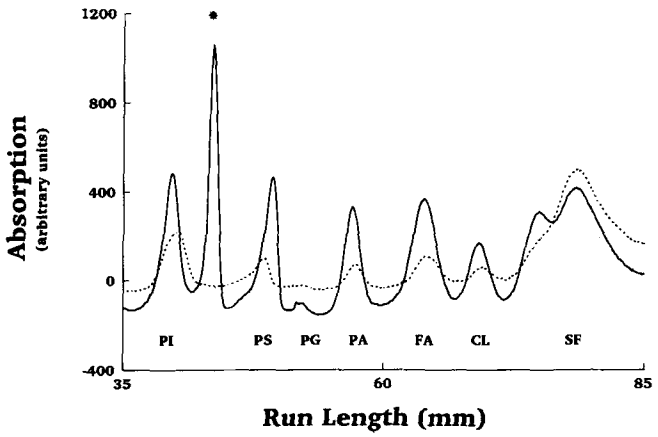

FIG. 2. High-performance thin-layer chromatogram of anionic phospholipids and fatty acids (FA) of the homogenate from pancreatic islets. The asterisk denotes an artifactual splitting of the phosphatidylinositol (PI) peak due to residual salt contamination. At lower sample loads, only the basic PI peak was observed (dotted chromatogram). Abbreviations as in Figure 1; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin.

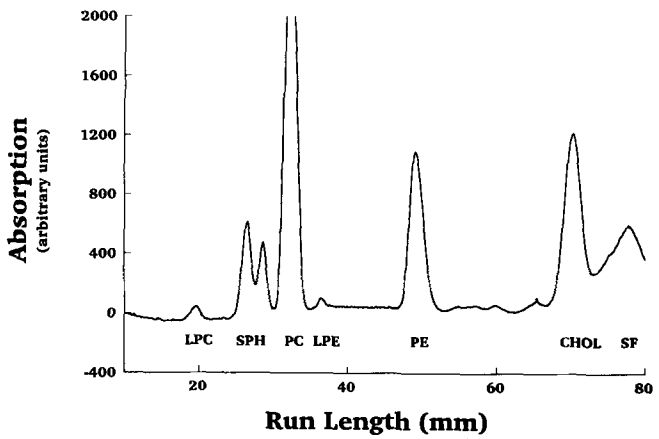

FIG. 1. High-performance thin-layer chromatogram of phospholipids and neutral lipids of the homogenate from glucose-stimulated pancreatic islets. LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; CHOL, cholesterol; SF, solvent front.

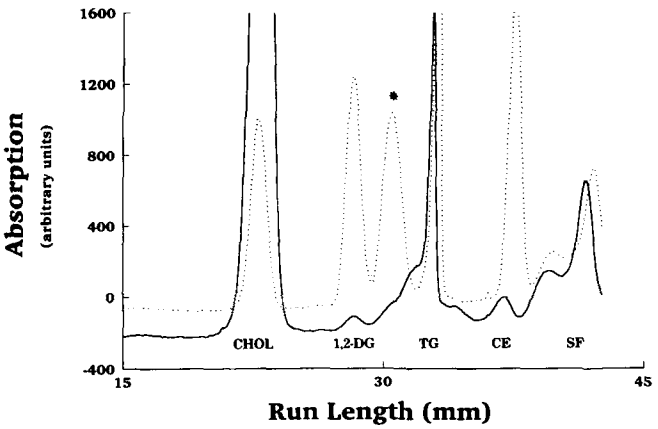

FIG. 3. High-performance thin-layer chromatogram of neutral lipids of the homogenate from glucose-stimulated pancreatic islets. The profile for the standards is given in the dotted chromatogram. The asterisk denotes 1,3-diacylglycerol (DG). The tailing of the triacylglycerol (TG) peak is caused by the multiple development system. Abbreviations as in Figure 1; CE, cholesteryl ester.

homogenate or subcellular fraction. Protein was determined according to McKnight (35) in aliquots which had not been subjected to lipid extraction. Values are presented as means ± SEM and were tested for statistical significance by Student's unpaired *t*-test.

RESULTS

Phospholipid and neutral lipid composition of homogenates of glucose-stimulated pancreatic islets in comparison with controls. The total phospholipid and neutral lipid content of pancreatic islets did not change significantly during a 5-min stimulation with 25 mM glucose as compared with islets maintained at a nonstimulatory glucose concentration (5 mM). Phospholipids represented 68.2% of the total lipid mass in unstimulated and 62.4% in stimulated islets; the respective values for neutral lipids were 31.8 and 37.6% (Table 2). Among the phospholipids, the molar percentage of two anionic phospholipids showed significant changes (Table 3): Phosphatidylinositol (PI), the most abundant anionic phospholipid, increased from 8.6 to 11.8 mol% ($P < 0.05$), and PA increased from 3.1 to 4.7 mol% ($P < 0.005$). Lysophospholipids, which are regarded by some authors as potential mediators in the induction of insulin secretion (2), showed no change in abundance during this short-term incubation. LPC, lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI) and lysophosphatidic acid (LPA) were resolved chromatographically. In both stimulated and resting islets, LPE and LPC represented 5% of their parent compounds, whereas LPI and LPA could not be found in any of the samples. In view of the increase in PA, a simultaneous increase in LPA might have indicated whether *de novo* synthesis was responsible for the increase in PA mass. However, the LPA peak in the standard chromatograms was not baseline-separated from the peak of phosphatidylserine (PS), and, therefore, small amounts of LPA (<10% of the PS peak area) may have escaped detection. LPG was also not separated from PS. However, in view of the low abundance (0.2 mol%) of PG, it appears unlikely that LPG was present in amounts similar to those of LPC and LPE. PC and PE were the most abundant phospholipids. In accordance with earlier reports on the phospholipid composition of pancreatic islets (see Table 1), the content of PC was 49.9 mol% and that of PE 21.2 mol%. The molar percentages were not significantly different in glucose-stimulated islets (48.4 and 18.6 mol% for PC and PE, respectively). SPH was resolved into two subfractions. The component with the lesser R_f value comprised 64% of the total SPH mass. The slower migration of this component probably reflects the presence of a hydroxyacyl group (2-hydroxylignoceric acid and 2-hydroxynervonic acid), as is known to occur in sphingolipids (31,36). The mitochondrial phospholipid, cardiolipin (CL), comprised only 1 mol% in the islet homogenates. This very low value is consistent with earlier data published on CL abundance in pancreatic islets (Table 1).

Among the neutral lipids of pancreatic islets, cholesterol (CHOL) was by far the major component (73 mol% of the neutral lipids). The contents of CHOL, triacylglycerol (TG) and unesterified fatty acids (FA) were unchanged after glucose stimulation. As the intensity of charring depends on acyl chain length and unsaturation, comparatively large SEM values were observed in the FA determinations. The sensitivity of the charring reaction permitted the detection of small amounts of DG present in pancreatic islets (Fig. 3). The peak areas of 1,2-DG were sufficient for quantitation, and no change could be seen in the glucose-stimulated islets (Table 4). 1,3-DG was present only in trace amounts.

Phospholipid and neutral lipid composition of homogenates and subcellular fractions of RINm5F cells. In homogenates of RINm5F cells, the relative contribution of phospholipids (71.6%) and neutral lipids (28.4%) relative to the total lipid mass (Tables 5 and 6) was the same as in resting pancreatic islets (68.2 and 31.8%, respectively). However, the lipid/protein ratio was significantly ($P < 0.01$) lower than in pancreatic islet tissue (136.5 vs. 228.9 nmol/mg). Although the pattern of the phospholipids in the homogenate of the RINm5F cells was similar to the one of the homogenates of pancreatic islets, three lipid

TABLE 2

Total Contents of Phospholipids and Neutral Lipids in Homogenates from ob/ob Mouse Pancreatic Islets After Incubation for 5 min at Low or High Glucose Levels[a]

	(nmol/mg protein)	
	Phospholipid content	Neutral lipid content
5 mM glucose	156.0 ± 7.2 (68.2)	72.9 ± 7.1 (31.8)
25 mM glucose	140.2 ± 6.3 (62.4)	84.6 ± 6.1 (37.6)

[a]The contents of phospholipid and neutral lipid represent the sum of each type of lipid determined. The numbers in parentheses denote the respective percentage of the total lipid mass. The data are means ± SEM of four (5 mM glucose) or six (25 mM glucose) experiments. Neither the phospholipid nor the neutral lipid content is significantly different between the two groups.

TABLE 3

Composition of Phospholipids in Homogenates from ob/ob Mouse Pancreatic Islets After Incubation for 5 min at Low or High Glucose Levels[a]

	(mol% of total phospholipid)	
	5 mM Glucose	25 mM Glucose
LPC	2.7 ± 0.3	2.3 ± 0.4
SPH1	5.4 ± 0.3	5.3 ± 0.4
SPH2	3.0 ± 0.2	2.8 ± 0.2
PC	49.9 ± 1.0	48.4 ± 1.1
LPE	1.1 ± 0.1	1.0 ± 0.1
PE	21.2 ± 1.0	18.6 ± 0.6
PI	8.6 ± 0.8	11.8 ± 1.1[b]
PS	4.1 ± 0.6	4.1 ± 0.2
PG	0.2 ± 0.0	0.2 ± 0.0
PA	3.1 ± 0.2	4.7 ± 0.3[c]
CL	0.9 ± 0.1	1.0 ± 0.1

[a]The data are means ± SEM of four (5 mM glucose) or six (25 mM glucose) experiments. Where indicated, values are significantly different to those obtained on islets incubated at low glucose levels ([b]$P < 0.05$), [c]$P < 0.005$). To calculate the mass of individual phospholipids, the tabulated mol% is to be multiplied by the phospholipid content of each group (see Table 2). For abbreviations, see Table 1.

TABLE 4

Composition of Neutral Lipids in Homogenates from ob/ob Mouse Pancreatic Islets After Incubation for 5 min at Low or High Glucose Levels[a]

	(mol% of total neutral lipid)	
	5 mM Glucose	25 mM Glucose
FA	17.2 ± 3.6	18.3 ± 2.5
CHOL	73.7 ± 4.3	72.9 ± 3.4
TG	7.8 ± 1.6	7.7 ± 1.2
1,2-DG	0.22 ± 0.06	0.18 ± 0.05
1,3-DG	Trace	0.0
CE	0.31 ± 0.10	0.22 ± 0.08

[a]The data are means ± SEM of four (5 mM glucose) or six (25 mM glucose) experiments. To calculate the mass of individual lipids, the tabulated mol% is to be multiplied by the neutral lipid content of each group (see Table 2). TG, triacylglycerol; DG, diacylglycerol; FA, unesterified fatty acids; CHOL, cholesterol; CE, cholesteryl esters.

TABLE 6

Composition of Neutral Lipids in Homogenates and Subcellular Fractions from RINm5F Cells[a]

	(nmol/mg protein)			
	Homogenate	Mitochondrial fraction	Microsomal fraction	Cytosolic fraction
FA	2.0 ± 0.4	17.2 ± 3.5	23.1 ± 2.2	3.4 ± 1.1
CHOL	30.8 ± 2.5	145.2 ± 13.8	78.2 ± 8.3	4.4 ± 0.9
TG (+ DG + CE)	6.0 ± 1.2	16.4 ± 4.9	12.3 ± 4.4	5.8 ± 1.6
Σ	38.8 (28.4)	178.8 (46.1)	113.6 (51.9)	13.6 (76.8)

[a]The data are means ± SEM of 4–5 experiments. The TG value also includes DG and CE. The numbers in parentheses denote the percentage of neutral lipids relative to the total lipid content. For abbreviations, see Table 4.

classes showed clear differences (Table 5): CL and PG were present at higher percentages (4.7 and 2.0 mol% in RINm5F cells vs. 0.9 and 0.2 mol% in islets), whereas PA was present only in trace amounts in RINm5F cells. As in pancreatic islets, SPH was resolved into two subclasses, and the component with the lesser R_f value was again predominant (74% of total SPH mass).

Among the subcellular fractions, the phospholipid/protein ratio was highest in the mitochondrial fraction and amounted to 208.9 nmol/mg, which is similar to the mitochondrial phospholipid content in other, nontumorous tissues (37,38). In the mitochondrial and microsomal fractions of the RINm5F cells, the neutral lipid percentage was higher than in the homogenates (46.1 and 51.4% vs. 28.4%), which was mainly due to a high CHOL content in these fractions (Table 6). Another interesting feature in addition to the high CHOL content was the high percentage of SPH in the mitochondrial fraction (total SPH 15.8 mol%), as mitochondrial membranes usually have a very low SPH content (39). The CL content of the mitochondrial fraction was 2.8-fold increased when compared to that of the homogenate (13.1 mol% vs. 4.7 mol%). The increased percentage of CL in the mitochondrial fraction was paralleled by a similar increase in PG from which CL is synthesized in mitochondria (Table 5). The mitochondrial fraction was the only one in which PA could be quantitated; in the homogenate and in the microsomal fraction, PA was present only in trace amounts (Table 5).

TABLE 5

Composition of Phospholipids in Homogenates and Subcellular Fractions from RINm5F Cells[a]

	(mol% of total phospholipid)			
	Homogenate	Mitochondrial fraction	Microsomal fraction	Cytosolic fraction
LPC	1.9 ± 0.3	2.6 ± 0.6	4.2 ± 1.0	6.1 ± 2.4
SPH1	5.7 ± 0.7	11.2 ± 0.6	14.2 ± 2.6	20.7 ± 3.7
SPH2	2.0 ± 0.2	4.6 ± 0.6	6.3 ± 1.4	8.4 ± 2.2
PC	52.5 ± 4.7	40.8 ± 5.7	39.2 ± 8.3	42.8 ± 6.3
LPE	2.7 ± 0.4	2.3 ± 0.8	3.8 ± 1.2	3.4 ± 1.8
PE	18.1 ± 1.3	10.5 ± 1.8	12.3 ± 3.9	4.8 ± 1.1
PI	6.5 ± 0.3	2.9 ± 0.6	4.1 ± 0.6	trace
PS	3.9 ± 0.5	6.1 ± 1.9	10.8 ± 3.6	8.9 ± 0.2
PG	2.0 ± 0.6	5.5 ± 1.7	1.0 ± 0.5	0.0
PA	trace	0.5 ± 0.3	trace	0.0
CL	4.7 ± 0.4	13.1 ± 2.6	4.1 ± 0.8	4.8 ± 2.3
Σ (nmol/mg)	97.7 (71.6)	208.9 (53.9)	105.1 (48.1)	4.1 (32.2)

[a]The data are means ± SEM of 4–5 experiments. The numbers in parentheses denote the percentage of phospholipids relative to the total lipid content. To calculate the mass of individual phospholipids, tabulated mol% is to be multiplied by the phospholipid content of each group. An amount was termed "trace" when the peaks were regularly visible, but below the limit of determination. For abbreviations, see Table 1.

Phospholipid and neutral lipid composition of homogenates and subcellular fractions of glucose-stimulated STZ/NA-induced islet cell tumors in comparison with controls. In most determinations of the lipid composition of the STZ/NA-induced islet cell tumors, the standard errors were larger than in the corresponding determinations with RINm5F cells or islets (Tables 7 and 8). The different sizes of the tumors, resulting in less homogenous samples are likely to contribute to this. Similar to the lipid distribution in RINm5F cells, a low content of PA in the homogenate (0.2 mol%) was seen. Considerable amounts of CHOL and SPH in the mitochondrial fraction were also reminiscent of the results obtained with RINm5F cells, even though the mass of CHOL in the mitochondrial fraction was clearly lower (71.8 nmol/mg in STZ/NA tumors vs. 145.2 nmol/mg in RINm5F cells). As in both other insulin secretory tissues, SPH consisted of two subfractions, of which the low-R_f component was predominant (65% of total SPH mass). A glucose stimulus (25 mM for 30 min) affected the molar percentages of three phospholipids, but none of the neutral lipids (Tables 7 and 8). The PC content in the homogenate was significantly lowered by 20% ($P < 0.05$). Practically the same reduction (25%; $P < 0.05$) was seen in the microsomal fraction (Table 7). Such a reduction may reflect PLA_2 activation in the course of insulin secretion (2), but no accumulation of LPC was seen in the homogenate and the microsomal fraction. However, there was a significant increase in LPC in the mitochondrial

TABLE 7

Composition of Phospholipids in Homogenates and Subcellular Fractions from STZ/NA Tumors[a]

	(mol% of total phospholipid)			
	Homogenate	Mitochondrial fraction	Microsomal fraction	Cytosolic fraction
LPC	4.2 ± 1.3	3.0 ± 0.5	4.2 ± 1.0	6.9 ± 1.0
LPC		5.0 ± 0.5[b]		
SPH1	8.3 ± 2.2	4.2 ± 0.9	6.0 ± 1.6	7.3 ± 2.7
SPH2	5.4 ± 0.9	3.2 ± 0.7	4.7 ± 1.4	5.4 ± 1.4
PC	51.2 ± 2.2	41.7 ± 2.5	52.8 ± 3.1	39.0 ± 7.2
PC	41.1 ± 2.5[b]		39.4 ± 2.6[b]	
LPE	1.4 ± 0.4	1.7 ± 0.2	1.9 ± 0.3	0.6 ± 0.3
PE	23.1 ± 4.8	29.0 ± 6.1	24.8 ± 5.1	24.7 ± 9.5
PI	7.6 ± 1.5	8.7 ± 1.9	7.7 ± 1.9	8.6 ± 1.2
PS	1.9 ± 0.4	2.5 ± 0.6	3.3 ± 0.7	4.2 ± 0.7
PG	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.0
PA	0.1 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	3.1 ± 1.7
PA			0.9 ± 0.2[b]	
CL	3.2 ± 0.4	4.8 ± 0.6	0.1 ± 0.1	0.2 ± 0.2
Σ (nmol/mg)	136.8 (67.4)	281.1 (73.4)	203.6 (56.0)	18.8 (52.5)

[a]The values that were not significantly different between stimulated (25 mM glucose for 30 min) and control tumors were treated as one group and are means ± SEM of six experiments. The numbers in parentheses denote the percentage of phospholipids relative to the total lipid content. To calculate the mass of individual phospholipids, the tabulated mol% is to be multiplied by the phospholipid content of each group. For abbreviations, see Table 1.
[b]Where significant differences occurred ($P < 0.05$), the value of the stimulated tumors is given in the line below the value of the control tumors; in that case each value is a mean ± SEM of three experiments.

fraction. Apparently due to the small size of the mitochondrial fraction and the comparatively low abundance of LPC, a similar change was not detectable in the homogenate. The same reasoning applies to the 4.5-fold increase in PA in the microsomal fraction ($P < 0.05$), which was not reflected in a significant change in PA in the homogenate.

TABLE 8

Composition of the Neutral Lipids in Homogenates and Subcellular Fractions from STZ/NA Tumors[a]

	(nmol/mg protein)			
	Homogenate	Mitochondrial fraction	Microsomal fraction	Cytosolic fraction
FA	14.7 ± 3.1	17.8 ± 4.4	27.3 ± 5.3	5.5 ± 1.2
CHOL	42.2 ± 5.3	71.8 ± 8.9	103.0 ± 18.4	9.6 ± 3.7
TG (+ DG + CE)	9.3 ± 2.3	12.5 ± 2.3	29.7 ± 9.3	1.8 ± 0.6
Σ	66.2 (32.6)	102.1 (26.6)	160.0 (44.0)	16.9 (47.6)

[a]There were no significant differences between the values from stimulated (25 mM glucose for 30 min) and control tumors; therefore, the mean values ± SEM for all six tumors are given. The TG value also includes DG and CE. The numbers in parentheses denote the percentage of neutral lipids relative to the total lipid content. For abbreviations, see Table 4.

DISCUSSION

The central observation in experiments conducted with pancreatic islets was the increase in PA and PI mass after a 5-min stimulation at a maximally effective glucose concentration (25 mM). The magnitude of the change in PA (+52%) was similar to the increase of radioactively-labeled palmitic acid in PA after a 30 min stimulation with 28 mM glucose reported recently by Konrad and colleagues (40). The paucity of data on the mass of PA (Table 1) is due to the low abundance of PA and the difficulty of separating PA from other phospholipids, particularly PE. The value for PA mass in islet tissue reported by Dunlop *et al.* (10) appears surprisingly high when recalculated on a nmol/mg basis (see Table 1). In conjunction with the increase in PA, the increase in PI mass in stimulated islets may indicate *de novo* synthesis of these lipids. *De novo* synthesis of PA and PI early in glucose stimulation has been proposed to occur by Farese *et al.* (41) and Dunlop and Larkins (42). However, generation of PA from PC through hydrolytic cleavage by a PLD is also possible, as recently shown by Metz and Dunlop (19).

De novo synthesis of PA and PI appears to be an attractive mode in view of the physiological role of pancreatic islets as fuel-sensors. PA synthesis connects glycolysis and FA metabolism and can relate both pathways to the generation of a second messenger. This may be 1,2-DG generated from PA by the action of a phosphohydrolase, but PA may also act as a signal transducer in its own right (43,44). It is conceivable also that PA through its mitogenic action (43) might act as a mediator of the proliferative effect of glucose on pancreatic B cells (45). Although the lack of a change in 1,2-DG noted in our study does not rule out a second messenger role for DG, it may suggest that PA could play a role in signal transduction rather than simply being a precursor of 1,2-DG. Constant levels of 1,2-DG during glucose-induced insulin secretion have also been reported by Wolf and colleagues (46) who measured 1,2-DG up to 20 minutes after the initiation of glucose stimulation using a DG-kinase assay. Most of the 1,2-DG measured in this study is likely to be located in the endoplasmic reticulum due to its role as an intermediate in the synthesis of phospholipids and TG (47). It is not clear whether a significant amount of 1,2-DG is available to serve as a second messenger at the plasma membrane level (48). Products of PLA_2 reactions form a second group of potential lipid mediators in glucose-induced insulin secretion (2,15). These include lysophospholipids and polyunsaturated FA, in particular arachidonic acid.

In the present study, we did not analyze FA compositions but only determined unesterified FA as a class. The amount of total FA we measured (12 nmol/mg protein) did not change upon glucose stimulation. The mass of arachidonic acid was earlier determined by Wolf and colleagues (17) and was found to be 2 pmol/islet after glucose stimulation, which is roughly equivalent to 4 nmol/mg protein. HPTLC resolved all relevant lysophospholipids, but the mass of one of the most abundant compounds, LPC, was found unchanged. This is somewhat in contrast to data by Turk and colleagues (21) who reported a decline in LPC mass from a rather high initial value of 5.5 to 0.5

pmol/islet during glucose stimulation. However, an increase in LPC that is confined to only one specific subcellular compartment can be overlooked when only homogenates are examined. In pancreatic B cells, the mitochondria represent only a minor compartment as the mitochondrial volume accounted for only 4% of the total B cell volume, as opposed to more than 20% in hepatocytes (49). The small size of the mitochondrial compartment in pancreatic B cell can also explain the low content of CL in the homogenate (1 mol%), as CL is exclusively located in the inner mitochondrial membrane.

The observation that the total phospholipid mass was not increased in stimulated islets may appear surprising in view of the numerous reports on enhanced phospholipid and neutral lipid synthesis during glucose-induced insulin secretion (3,5,6,8,41). However, the data are in accordance with those of Montague and Parkin (9), who found that the phospholipid (230 nmol/mg) and cholesterol (60 nmol/mg) contents of guinea-pig islets were unchanged after glucose stimulation. Turk *et al.* (21) also found a slightly reduced content of PC (150 vs. 130 pmol/islet as determined by FA mass) after 30 min of glucose stimulation, whereas all other major phospholipids were slightly, but not significantly, reduced. Thus, activation of a particular lipid metabolic pathway during glucose stimulation does not necessarily result in an accumulation of a presumptive mediator. Alternatively, the analyses of the lipid pattern in homogenates may not be sufficient to elucidate transient changes restricted to one subcellular compartment. The larger amounts of tissue available from the RINm5F insulinoma cell line or from STZ/NA-induced tumors permit the analysis of lipids from subcellular fractions. The large amounts of SPH and CHOL, as well as of unesterified FA, found in the mitochondrial fraction are consistent with observations made on hepatoma cells (39,50,51), indicating a loss of the typical pattern of biosynthesis and intracellular translocation of these lipids. The predominance of hydroxyacyl species of SPH, which was most marked in RINm5F cells, was a common characteristic of all the insulin secretory tissues studied here. By contrast, liver, kidney and erythrocytes contained only low levels of the hydroxylated SPH species (I. Rustenbeck, unpublished data). Compared to normal islets in the resting state, RINm5F cells contained much less PA and larger amounts of CL and of its precursor, PG, in the homogenates. More detailed comparisons between the three types of tissues should only be made with caution, as the different methods used to prepare the tissues (collagenase digestion vs. trypsination vs. microdissection) may affect observed lipid patterns. Bearing this in mind, it nevertheless appears that the abovementioned similarities between the lipids of RINm5F cells and those of other tumorous tissues somewhat limit the usefulness of this cell line to further investigate the possible role of lipid mediators in insulin secretion. One could even speculate that the alterations in the lipid metabolism might actually contribute to the defective secretory responsiveness of these cells in addition to the well-known defective recognition of glucose (25).

In contrast to RINm5F cells, STZ/NA tumors are responsive to changes in glucose concentration in the physiological range, although the insulin secretory responsiveness decreases with increasing tumor size (27). The subcellular distribution of lipids appeared to be less deranged than in the RINm5F cells. The localization of the increase in PA in the microsomal fraction would be consistent with *de novo* synthesis of PA. The increase in LPC in the mitochondrial fraction might result from an accumulation of Ca^{2+} in the mitochondria during prolonged glucose stimulation (52), leading to an activation of mitochondrial PLA_2 (53). These changes need not necessarily be related to stimulus–secretion coupling, but they demonstrate that activation of PLA_2 or PLD in a small subcellular compartment may not be detectable in tissue homogenates. Therefore the data obtained with islet homogenates suggest a second messenger role for PA, but at the same time do not rule out such a role for 1,2-DG or LPC.

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Effect of Dietary Fat on Colonic Protein Kinase C and Induction of Aberrant Crypt Foci[1]

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A major objective of the present study was to determine whether a high-fat diet affects early events during colon carcinogenesis. Female Sprague-Dawley rats were injected with saline or azoxymethane (20 mg/kg) and fed either a normal (5% corn oil w/w) or a high (5% corn oil and 15% beef tallow w/w) fat diet. To assess the effect of a known tumor-promoting diet on the early events of neoplastic transformation, Study 1 examined the induction and growth of aberrant crypt foci (ACF) as well as of proliferative indices. The total number of ACF were similar in both groups even after 8 wk of dietary treatment; however, ACF with accelerated growth characteristics (≥4 crypts/focal lesion) were more prevalent ($P \leq 0.05$) in the colons of animals fed the high-fat diet. Metaphase arrest cells and 5′-bromo-2′-deoxyuridine labelled cells showed no appreciable response to dietary changes. To determine whether changes in colonic signal transduction pathways represent an early response to dietary modification, Study 2 evaluated the activity of protein kinase C (PKC), proliferative indices and changes in phospholipid fatty acid profiles. In comparison to the normal fat group, the colons of high-fat fed animals exhibited higher ($P \leq 0.05$) membranes and lower soluble PKC activity; however, proliferation patterns of these colons were not altered. Changes in the membrane lipid composition were minor; however, an increase in the phosphatidylcholine/phosphatidylethanolamine ratio and in 20:4n-6 was noted. Our results demonstrate that in comparison to a normal-fat diet, a high-fat diet stimulated the growth of a population of ACF, i.e., preneoplastic lesions leading to advanced growth characteristics. In addition, a high-fat diet exerted a marked influence on total, cytosolic and membrane associated PKC activities. The findings suggest that modulation of PKC may play a critical role at the early stages of colon carcinogenesis.

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Epidemiological and animal studies have contributed a great deal to our understanding of the role of dietary components in the etiology and prevention of colon cancer (1–6). Several studies have demonstrated that rodent diets containing 20% fat by weight or more from sources such as corn oil, safflower oil, beef fat, lard or a combination thereof, have a promotional effect on chemically-induced colon carcinogenesis (3–6). However, this promotional effect is not seen when the dietary fat is derived from fish oil or coconut oil (4,7).

Although the effect of fat has been well-documented, the exact mechanism by which the high-fat diet exerts this promotional effect on colon carcinogenesis remains poorly understood. Several mechanisms have been offered to explain the tumor-modulating effect of high-fat diets. One such mechanism suggests that a high-fat diet increases the level of luminal free fatty acids and bile acids that may reach the colon and irritate the mucosa, thus enhancing proliferation of colonic epithelial cells (8–10).

It has also been proposed that membrane phospholipid composition may regulate the activity of protein kinase C (PKC), an enzyme known to play a pivotal role in cell growth and differentiation (11,12). Alterations in PKC activity may thus affect the carcinogenic process (13).

The main objectives of the present investigation were to assess the effect of a high beef tallow diet with known tumor-promoting activity on the number and growth features of aberrant crypt foci (ACF) which are considered preneoplastic lesions. We also assessed the effect of this diet on phospholipid composition, proliferative indices and PKC activity of rat colonic mucosa. Feeding a high beef tallow diet to rats resulted in an increased number of aberrant crypt foci which exhibited advanced growth features compared to those seen with animals on a normal fat diet. In addition, the high beef tallow diet exerted a distinct effect on the membrane lipid composition and PKC activity of rat colonic mucosa.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Campus Breeding, Department of Animal Care, University of Manitoba, Winnipeg, Canada), approximately 5–6 weeks old, were acclimated for one week prior to the experiments. The animals were housed in wire cages using a 12:12 h light/dark cycle. Temperature and humidity were controlled at 22°C and 50%, respectively. Animals had access to food and water *ad libitum*. All animals were cared for according to the guidelines of the Canadian Council of Animal Care.

Diets. The formulated diets were based on a modified AIN-76A diet (14,15) as described in Table 1. For the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate.

Study 1: Preneoplastic lesions and proliferative patterns. Animals were injected with a single dose of azoxymethane (AOM), 20 mg/kg (Sigma Chemical Co.,

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Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; ATP, adenosine triphosphate; BrdU, 5′-bromo-2′-deoxyuridine; DAG, diacylglycerol; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PS, phosphatidylserine.

TABLE 1

Diet Composition

Ingredients	Normal fat[a] (%)	High fat[b] (%)
Dextrose	50.0	35.0
Casein	20.0	22.7
Dextrin	15.0	10.5
Corn oil	5.0	5.0
Beef tallow	0.0	15.0
α-Cellulose	5.0	5.9
AIN-76 mineral mix	3.5	4.1
AIN-76 vitamin mix	1.0	1.2
DL-Methionine	0.3	0.4
Choline bitartrate	0.2	0.2
Total grams	100.0	100.0
Caloric density (kcal/g)	3.8	4.5
% Calories from fat	11.6	40.6
% Calories from linoleic acid	7.1	7.0

[a]Modified AIN-76A diet composition.
[b]Diet based on modified AIN-76A adapted for a high-fat intake.

St. Louis, MO) and placed on the normal fat (control) diet for 1 wk. Animals were randomly allocated into dietary groups (8 animals/group) and fed either the high or normal fat diet for 8 wk. Two hours and one hour prior to termination by carbon dioxide asphyxiation, animals received an i.p. injection of colchicine, 1 mg/kg body weight (Sigma) or 5′-bromo-2′deoxyuridine (BrdU), 30 mg/kg body weight (Sigma). Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from caecum to anus, and fixed in 70% ethanol. Colons were assessed for ACF and proliferative indices.

Quantification of ACF. Following the protocol established by Bird (16), the fixed colons were stained in a 0.02% solution of methylene blue (Sigma); then using a light microscope, colons were assessed for the number and growth of ACF. The criteria used to identify a focus of ACF included: (i) increased size, (ii) thicker epithelial cell lining and (iii) increased pericryptal zone relative to normal crypts (17). Visualization and quantification of the number and crypt multiplicity of ACF in the entire colon were done as previously described (16,18). In order to determine crypt multiplicity, the number of crypts in each focus was recorded. To determine the distribution of ACF, the colon was divided into three sections. R represented the first 4 cm from the rectal end, M was the next 4 cm and C was the next 3–4 cm from M.

Measurement of mitotic and BrdU indices. Colonic tissue was processed for histology and mitotic proliferation as assessed by scoring crypt sections for colchicine (Sigma) arrested mitotic figures. BrdU immunohistochemistry was performed as described for proliferating cell nuclear antigen (PCNA) immunohistochemistry with the additional step of DNA digestion by 2N HCl treatment for 1 h following rehydration. The anti-BrdU monoclonal antibody (Becton-Dickinson, San Jose, CA) 1:40 dilution in antibody diluting buffer was applied to tissues for 1 h. For determination of the BrdU labeling index or mitotic index, ten well-orientated crypts were evaluated in which the base, lumen, and top of the crypts could be seen displaying a U-shaped configuration. The number and the position of the positively identified cells in each crypt column were recorded in terms of serial position counting upwards from position 1, at the base of the crypt up to the mouth of the crypt. The BrdU labelling index and mitotic index were calculated as the number of positive cells per crypt divided by the total number of cells per crypt multiplied by 100.

Study 2: Colonic PKC activity, cells exhibiting PCNA and mucosal lipid composition. Animals received a single saline injection and were then placed on the normal fat diet for one week. Animals were randomly allocated into dietary groups (10 animals/group) and fed either the high or the normal fat diet for 4 wk, after which time they were killed by carbon dioxide asphyxiation.

Extraction, separation and assay of PKC from rat colonic tissue. Rat colonic PKC activity was measured according to the PKC assay previously described (19) with some modifications. Briefly, colons were removed, flushed with cold Krebs Ringer solution, slit longitudinally and placed on a 0°C cooled surface to collect colonic mucosal scrapings. The tissue was homogenized in 25 mM Tris buffer (pH 7.5) containing 0.25 mM phenylmethylsulfonyl fluoride, 15 mM ethyleneglycol-*bis*(ß-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 0.25 M sucrose and 10 µg/mL trypsin inhibitor. The homogenized mixture was centrifuged at 100,000 × *g* for 60 min. The supernatant, which represents the soluble fraction, was applied to a pre-equilibrated DEAE-sephacel column. The column was washed with two bed volumes of Buffer A (lacking Triton-X 100) and eluted with a 25 mM Tris Buffer B containing 0.25 mM PMSF, 15 mM mercaptoethanol and 0.15 mM NaCl. The pellet was resuspended and homogenized in Buffer A with 0.5% Triton-X 100, centrifuged at 100,000 × *g* for 60 min, and its supernatant, which represents the particulate fraction, was partially purified as described for the soluble fraction. All column procedures were carried out at 4°C unless otherwise noted. Activity was determined by comparing the transfer of ^{32}P from γ-[^{32}P]adenosine triphosphate (ATP) (NEN, Mississauga, Ontario, Canada) to a histone protein in both the presence and absence of phosphatidylserine (PS) and diacylglycerol (DAG). All reaction mixtures contained 25 mM Tris buffer (pH 7.5) with 10 mM $MgCl_2$, 740 µg/mL histone, 2 mM $CaCl_2$, with (PS–DAT and calcium-dependent] or without (non-PS–DAG-dependent) 80 µg/mL PS and 8 µg/mL DAG. A 75-µL aliquot of the reaction mixture was added to 25 µL of column eluate; the reaction was then initiated by addition of 10 µL of γ-[^{32}P]ATP (0.45 µCi/10 µL) and allowed to incubate at 37°C for 5 min. Reactions were then quenched in an ice bath, and then 50 µL of the mixture was spotted onto small squares of P81 phosphocellulose paper. The paper was immersed in 75 mM phosphoric acid, washed with ddH_2) and the dried squares were counted for radioactivity on a Beckman LS6000TA Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA). Protein content was assayed using the Coomasie Blue protein assay (Sigma).

Measurement of PCNA indices. Colonic tissue was embedded in paraffin wax, and 5-µm thick sections were

processed for immunohistochemistry employing the unlabelled antibody bridge method and the Universal Peroxidase kit from Signet Laboratories (ID Labs Inc., London, Ontario, Canada); the method is similar to that described by Richter *et al.* (20). Tissue sections were deparaffinized and then flooded with normal goat serum and incubated for 20 min to block nonspecific binding. The anti-PCNA monoclonal antibody (Dimension Laboratories, Inc., Mississauga, Ontario, Canada) diluted with antibody diluting buffer (1:40) was applied to tissue sections, and the slides were subsequently incubated for 1 h. Each tissue section was then sequentially incubated with anti-mouse IgG (antibody bridge) and mouse IgG peroxidase (labeling agent). The peroxidase reaction was initiated by immersing the slides in 0.06% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in PBS to which 0.03% H_2O_2 had been added immediately prior to use. Finally, the slides were lightly counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Ottawa, Ontario, Canada). All incubations were carried out in a humidified chamber at room temperature, and between incubations slides were extensively washed with PBS. The PCNA labelling index was determined by identifying ten well-orientated crypts in which the base, lumen and the top of the crypts could be seen displaying a U-shaped configuration. The number and the position of the labelled cells in each crypt column were recorded in terms of serial position counting upward from position 1, at the base of the crypt up to the mouth of the crypt. The PCNA labelling index was calculated at the number of positive cells per crypt divided by the total number of cells per crypt multiplied by 100.

Analysis of colonic lipids. Animals were killed by CO_2 asphyxiation and their colons removed rapidly and rinsed in ice-cold PBS. Colons were slit open longitudinally, freed from all contents, laid flat, and mucosa was scraped with a microscope glass slide. Lipids were extracted as described by Folch *et al.* (21) using chloroform/methanol (2:1, vol/vol). Phospholipids were separated by thin-layer chromatography using Silica Gel Merck 60 (BDH Inc., Toronto, Ontario, Canada) precoated plates. All major phospholipids were clearly separated following development of the plates in chloroform/methanol/acetic acid/water (50:37.5:3:1.5, by vol). Lipid bands were visualized with 2′7′-dichlorofluorescein and identified by comparison of their migration rate with those of standard phospholipids (Serdary Research Laboratories Inc., London, Ontario, Canada). Bands corresponding to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were scraped from the plates and the lipid fractions were directly transmethylated with 6% sulfuric acid in methanol for 2 h at 80°C in the presence of heptadecenaoic acid (17:0) as internal standard. Following incubation, the reaction mixture was cooled and diluted with water (1 mL) and petroleum ether (2 mL). The upper phase containing the fatty acid methyl esters was removed, dried under pure nitrogen, and reconstituted in small volumes of hexane. Reconstituted fatty acid methyl esters were analyzed by gas–liquid chromatography (Varian Star 3400; Varian Instruments, Palo Alto, CA) using a fused Omega Wax capillary column (30 × 0.25 μm) held at 160°C for 1 min, then ramped to 220°C at 2°C per min, and then held for 20 min using helium as the carrier gas.

Statistical analyses. Statistical analysis of the data was by Student *t*-test to compare group means using the SAS statistical software for microcomputers (SAS Institute, Inc., Cary, NC). A P value of <0.05 was considered significant.

RESULTS

The body weights of normal-fat and high-fat fed animals were similar throughout the experiment in both carcinogen and saline treated animals (Fig. 1); however, after 4 wk of dietary treatment, the body weight of animals fed the high-fat diet was greater, though not statistically significant, from the normal-fat diet (carcinogen: 273.1 ± 12.9 vs. 243.4 ± 5.9; saline: 220.0 ± 5.5 vs. 194.4 ± 11.2, respectively).

Study 1: Preneoplastic lesions and proliferative patterns. Dietary changes did not alter crypt height or the size of the proliferative zone in either metaphase arrest or BrdU labelling identification (results not shown). Although the number of cells in S-phase as measured by the BrdU labelling index were slightly greater in the high-fat fed animals both in the mid (3.7 ± 0.7 vs. 3.1 ± 0.6) and rec-

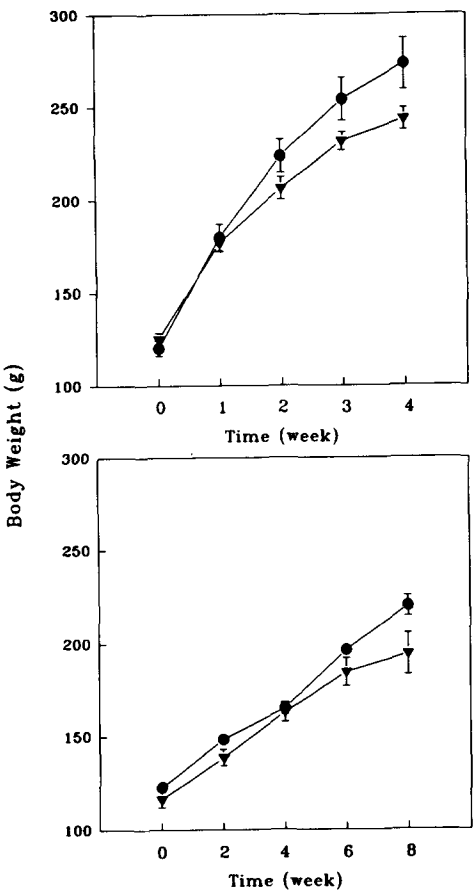

FIG. 1. Initial body weight and body weight gained in rats fed the high-fat (circle) or the normal fat (triangle) diets, and injected with saline in Study 2 (top panel) or AOM in Study 1 (bottom panel). Data represent mean ± SEM.

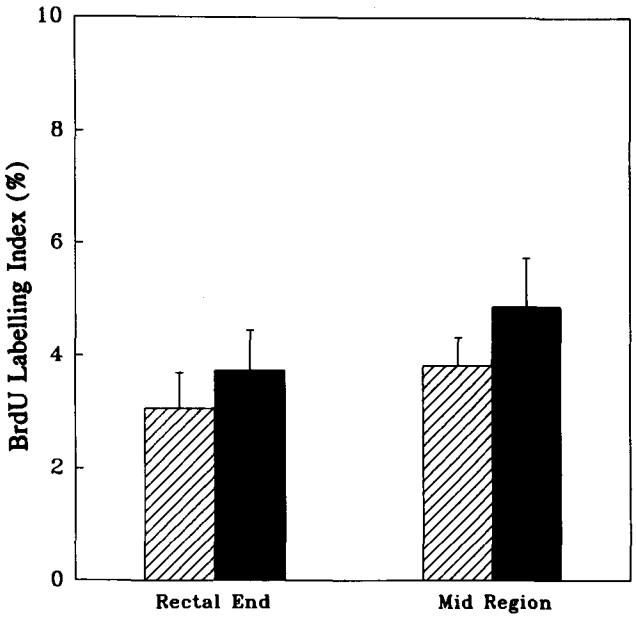

FIG. 2. Comparison of S-phase cells in carcinogen-treated colons between normal-fat at (hatched bar) and high-fat (filled bar) diets in the mid and in the rectal colonic regions as measured by the 5′-bromo-2′-deoxyuridine labelling index (BrdU). Column and bar represent the mean ± SEM.

tal (4.9 ± 0.9 vs. 3.8 ± 0.5) region, the changes did not reach statistical significance (Fig. 2). Similarly, mitotic activity, as measured by the number of cells arrested in metaphase, was greater, though not significantly, in the rectal (7.4 ± 1.6 vs. 5.4 ± 1.2) and mid (6.0 ± 0.9 vs. 5.6 ± 1.1) colonic mucosa of animals fed the high-fat diet (Fig. 3).

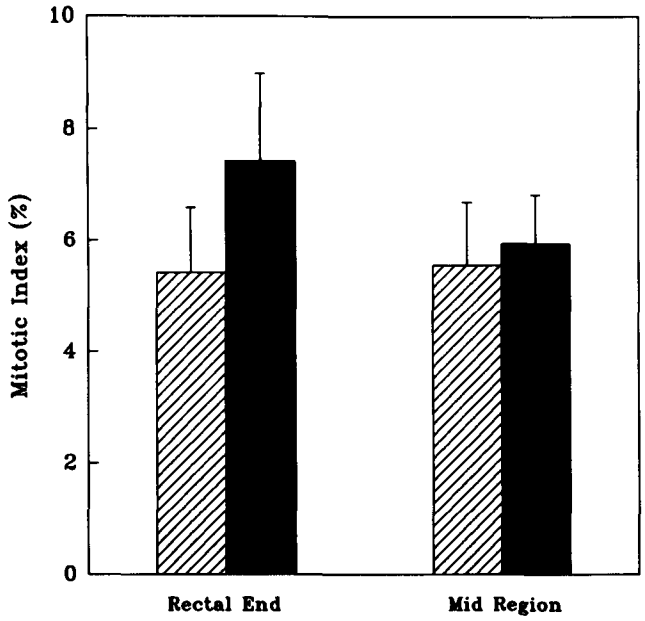

FIG. 3. Comparison of metaphase cells in carcinogen-treated colons between normal fat (hatched bar) and high-fat (filled bar) diets in the mid and in the rectal colonic regions as measured by the mitotic index. Column and bar represent the mean ± SEM.

The total number of ACF per colon (no. foci/colon) did not significantly differ between the two dietary groups although the mean multiplicity (no. AC/focus) was elevated in animals fed the high-fat diet (Table 2). The number of foci with crypt multiplicities of 1, 2 or 3 were not significantly altered by increased dietary fat intake. Interestingly, a significant increase in the population of ACF with advanced growth features (AC ≥ 4) was noted in response to high-fat consumption. A comparative distribution of these advanced lesions along the colon is shown in Table 2. In spite of an increase in the number of ACF with advanced growth features in the high-fat group, the relative distribution of ACF along the length of the colon was not affected by dietary treatment (Table 2).

Study 2: Colonic PKC activity, cells exhibiting PCNA, and mucosal lipid composition. The effect of the high-fat diet on the PCNA labelling index was not significantly different from that of the normal-fat diet in either the rectal or mid region of the colon (22.9 ± 1.1 vs. 19.2 ± 1.5; 22.0 ± 1.3 vs. 18.8 ± 1.0). However, a trend toward an elevated labelling index in the crypts of high-fat fed animals was observed which was similar in both the rectal and mid region of the colon (Fig. 4).

The activity of membrane (particulate) PKC was significantly higher (1.7 ± 0.4 vs. 0.7 ± 0.1) in the colonic mucosa of animals fed the high-fat diet (Fig. 5). Conversely, the PKC activity measured in the cytosolic (soluble) fraction was significantly lower (0.1 ± 0.0 vs. 4.0 ± 1.4) in the high-fat relative to the normal-fat fed animals. Total PKC activity was not significantly altered (normal; 4.6 ± 1.4 vs. high; 1.8 ± 0.3) by dietary changes.

The two major phospholipid classes of colonic mucosa were also quantified. Compared to the normal-fat diet group, the colons from animals fed the high-fat diet contained less PE resulting in a greater PC-to-PE ratio

TABLE 2

Effect of Dietary Fat on the Number and Growth Characteristic of Aberrant Crypt (AC) Foci[a]

	Normal fat	High fat
No. foci/colon	169.7 ± 24.4	185.5 ± 25.5
No. AC/focus	2.41 ± 0.07	2.79[b] ± 0.13
AC 1	30.7 ± 4.0	28.0 ± 6.7
AC 2	69.0 ± 9.3	67.3 ± 12.2
AC 3	44.5 ± 7.42	46.2 ± 7.3
AC ≥ 4	25.5 ± 6.61	44.0[b] ± 4.3
% Distribution of AC ≥ 4[c]		
R	34	30
M	40	43
C	26	27

[a]Mean ± SE, n = 8 animals/group. AC 1 to AC ≥ 4 = foci consisting of 1 to ≥4 AC.
[b]Significantly different from the corresponding normal fat group.
[c]Percent distribution of foci with four or more aberrant crypts along the divisions of the colon: R, the first 4 cm from the rectal end; M, the next 4 cm; the next 3–4 cm from M.

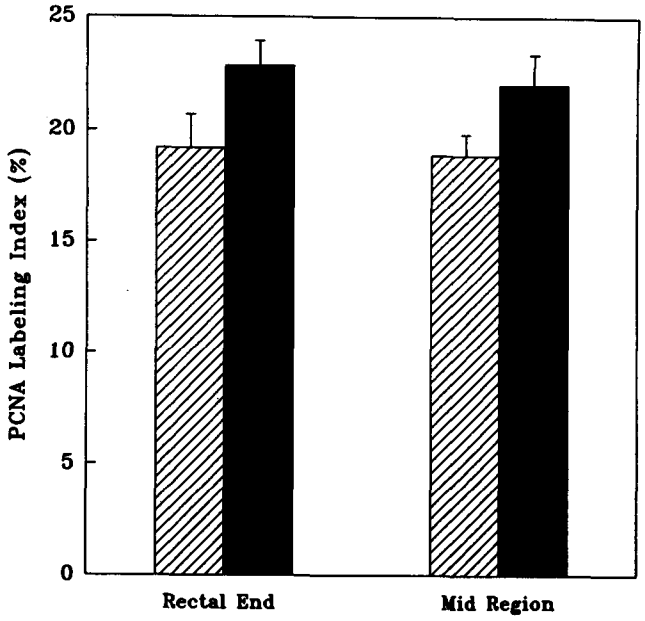

FIG. 4. Comparison of cycling cells in saline-treated colons between normal-fat (hatched bar) and high-fat (filled bar) diets in the mid and in the rectal colonic regions as measured by the proliferating cell nuclear antigen (PCNA) labelling index. Column and bar represent the mean ± SEM.

(Table 3). Generally the differences between the normal- and high-fat diet groups with respect to fatty acid composition of PC and PE were minor. The percentage of 16:0, 18:0 and 20:4n-6 in the PC fraction (Table 4) and 20:4n-6 in the PE fraction (Table 5) was higher in the mucosa of animals fed the high-fat diet.

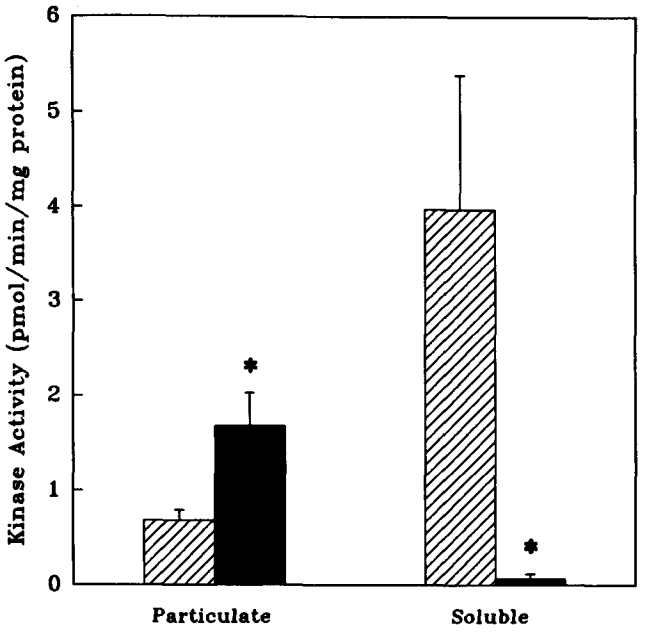

FIG. 5. Effect of normal-fat (hatched bar) and high-fat (filled bar) feeding on protein kinase C activity in both the particulate (membrane) and the soluble (cytosolic) fraction. Each column and bar represent the mean ± SEM, where an asterisk indicates a significant difference from the corresponding normal fat group $P \leq 0.05$.

TABLE 3

Effect of Feeding a Normal vs. a High Level of Dietary Fat on the Phospholipid Profile of the Colonic Epithelium[a]

Fatty acids	Normal-fat diet	High-fat diet
PC μg/g wet wt	829 ± 104	671 ± 64
PE μg/g wet wt	517 ± 156	300 ± 16
PC/PE ratio	1.8 ± 0.3	2.3 ± 0.3

[a]Values represent means ± SEM (n = 5). PC, phosphatidylcholine; PE, phosphatidylethanolamine.

TABLE 4

Effect of Feeding a Normal vs. a High Level of Dietary Fat on the Fatty Acid Profile of the Phosphatidylcholine Fraction[a]

Fatty acids	Normal-fat diet	High-fat diet
	% Fatty acid composition[b]	
14:0	1.2 ± 0.1	1.0 ± 0.1
16:0	43.7 ± 0.6	38.5 ± 0.5[e]
16:1	0.8 ± 0.02	0.5 ± 0.0[e]
18:0	17.2 ± 0.4	20.8 ± 0.8[d]
18:1n-9	11.9 ± 0.9	12.6 ± 0.5
18:2n-6	6.2 ± 0.4	5.4 ± 0.3
18:3n-6	nd	nd
20:1n-9	0.3 ± 0.1	0.5 ± 0.0
20:3n-6	1.6 ± 0.1	1.1 ± 0.0[c]
20:4n-6	12.6 ± 0.5	15.3 ± 0.9
20:5n-3	0.2 ± 0.2	trace
22:1n-9	0.6 ± 0.1	0.2 ± 0.0[d]
22:4n-6	1.4 ± 0.4	2.5 ± 0.3
22:5	0.8 ± 0.3	0.3 ± 0.03
22:6n-3	1.5 ± 1.1	1.2 ± 0.2
% Saturated	62.1 ± 0.3	60.3 ± 0.1[d]
% Monounsaturated	13.5 ± 1.1	13.8 ± 0.5
% Polyunsaturated	24.3 ± 1.2	25.9 ± 0.4

[a]Values represent means ± SEM (n = 5).
[b]The weight percent was calculated by dividing the weight of the fatty acids by the total fatty acids content and multiplying by 100; nd, not detectable.
[c]Significantly different from animals fed the normal 5% fat diet; $P < 0.05$; [d]$P < 0.01$; [e]$P < 0.001$.

DISCUSSION

In the present investigation we explored the possibility that PKC plays an important role in colon carcinogenesis and that the activity of this enzyme is affected by a high-fat diet which is known to exert a tumor-promoting effect. In Study 1, we found that a high-fat diet that stimulated the growth of a select population of ACF did not enhance proliferative indices of the colonic epithelium measured by two different procedures. Study 2 was conducted to determine whether the high-fat diet would affect PKC activity, phospholipid composition and proliferative indices measured by assessing cells exhibiting PCNA. Our data show that feeding a high-fat diet increased the colonic

TABLE 5

Effect of Feeding a Normal vs. a High Level of Dietary Fat on the Fatty Acid Profile of the Phosphatidylethanolamine Fraction[a]

Fatty acids	Normal-fat diet	High-fat diet
	% Fatty acid composition[b]	
14:0	0.4 ± 0.0	0.4 ± 0.0
16:0	12.3 ± 2.1	12.2 ± 0.0
16:1	1.2 ± 0.5	0.8 ± 0.4
18:0	33.4 ± 7.9	28.3 ± 0.7
18:1n-9	14.7 ± 1.1	13.0 ± 0.5
18:2n-6	4.5 ± 0.6	3.7 ± 0.2
18:3n-6	0.4 ± 0.1	0.5 ± 0.1
20:1n-9	0.6 ± 0.1	0.3 ± 0.1
20:3n-6	1.9 ± 0.3	1.5 ± 0.2
20:4n-6	20.7 ± 5.4	26.8 ± 1.1
20:5n-3	0.3 ± 0.2	0.3 ± 0.2
22:1n-9	0.2 ± 0.1	0.2 ± 0.0
22:4n-6	5.4 ± 1.3	7.5 ± 0.1
22:5	0.4 ± 0.0	0.6 ± 0.0[c]
22:6n-3	3.7 ± 0.3	3.9 ± 0.1
% Saturated	46.1 ± 5.9	40.9 ± 0.7
% Monounsaturated	16.7 ± 1.0	14.3 ± 0.8
% Polyunsaturated	37.3 ± 6.3	44.8 ± 1.0

[a]Values represent means ± SEM (n = 5).
[b]The weight percent was calculated by dividing the weight of the fatty acids by the total fatty acids content and multiplying by 100.
[c]Significantly different from animals fed the normal 5% fat diet: $P < 0.01$.

membranous (particulate) PKC activity and decreased the cytosolic (soluble) PKC activity when compared to animals fed a normal fat diet. A major effect of the high-fat diet on the lipid composition of the colonic mucosa was a decreased PE content and an increased percentage of 20:4n-6 in both the PC and PE fractions.

It is recognized that colon carcinogenesis is a multistep process which includes sequential selection and propagation of preneoplastic lesions. Aberrant crypt foci are present in carcinogen-treated rodent colons as well as in human colons with colon cancer in patients at high risk for colon cancer development (22–26). Several studies aimed at investigating the genotypic, morphologic and growth features of ACF have supported the contention that ACF are preneoplastic lesions (23–25,27). Moreover, a colon harboring a greater number of ACF with advanced growth features is at greater risk for developing the disease than one with fewer advanced ACF (28–30). The ability of the high-fat diet to increase the number of ACF with higher crypt multiplicity is compatible with the notion that this diet enhances the carcinogenic process; in the present investigation the high-fat diet exerted this effect by eight weeks of dietary treatment.

The high-fat diet did not significantly alter the proliferative activity of the colonic epithelium as compared to the normal-fat diet in either study regardless of the assessment used. Generally, assessment of S-phase cells either by enumerating the cells which incorporated BrdU or [^{3}H]thymidine has been advocated to estimate the risk of colon cancer development (31,32). It is known that cell replication is a complex multistep process that can be interrupted or stagnated at several stages by endogenous and exogenous factors (33,34) and that depending on the method of assessment different stages of the cell cycle are enumerated. We employed three different methods to evaluated proliferation of the colonic mucosa. The BrdU labelling index assesses the percentage of cryptal cells engaged in DNA synthesis, whereas the metaphase arrest technique allows enumeration of cells actually undergoing mitosis (35,36). There are only a few studies which have measured both parameters in the same colon. In the present study we found that both parameters were slightly increased in the high-fat fed animals. The assessment of cells exhibiting PCNA allows the identification of cycling cells. The antigens detected include various naturally occurring cell cycle related proteins (35). As a result, PCNA labelling indices tend to be greater than the BrdU labelling indices, and it has been suggested that this may be due to the gradient in PCNA expression that enlarges the window of reactivity (37). Although this method of assessment is in its early stages of development, several studies have reported excellent correlations between BrdU, [^{3}H]thymidine and PCNA proliferative indices (17,37). It has been suggested that PCNA immunohistochemistry may be a superior technique for determining proliferative status as this technique circumvents the use of radioactive ([^{3}H]thymidine) or cytotoxic (colchicine, BrdU) chemicals while still providing spatial and conformational information not available with flow cytometric analysis. The PCNA methodology is therefore ideal in situations where enzymatic and proliferative measurements are to be evaluated on the same specimen. In our experiments it was apparent that the PCNA labelling index tended to be greater in the colons of animals fed the high-fat diet, which was in accordance with the trends in the BrdU and mitotic indices assessed in the carcinogen-treated colons. The inability of the high-fat diet to significantly increase the proliferative indices of the colonic epithelium and its ability to accelerate the growth of a selected ACF suggests that the high-fat diet may be exerting a promotional effect on colon carcinogenesis by mechanisms other than stimulating colonic cell proliferation.

PKC is known to exist in various isoforms which exhibit differences in co-factor dependence and activator specificity (38,39). The ability of PKC to remain associated with the membrane as the active form may depend on the lipid composition of that membrane. It was apparent that membrane PKC activity was higher in the colonic mucosa of animals fed the high-fat diet suggesting that this diet may have increased the turnover rate of PKC. The lower amount of cytosolic PKC activity may demonstrate enhanced translocation of the enzyme to the membrane. This observation is compatible with the findings of others who have reported that tumor promoters appear to increase the translocation of cytosolic PKC to the particulate fraction (40). Furthermore, a study investigating the role of dietary fat and PKC in skin carcinogenesis found that activity in the particulate fraction increased twofold, and the soluble PKC activity decreased dramatically in animals consuming a high-fat diet (41). PKC has been recognized as having an important role in the regulation of cell growth and

differentiation (11,12). It has been established that PKC is a receptor for phorbol ester tumor promoters (42), and activation of this enzyme has been reported in response to bile acids, which are known to promote colon carcinogenesis (43). The enzyme has been implicated in imparting drug resistance (44) and is thought to play a role as a tumor suppressor gene (45). These findings taken together illustrate the possible importance of this enzyme in the pathogenesis of colon cancer. This has also led to the suggestion that PKC may serve as a convenient intermediate marker in the evaluation of intervention studies (43,46). However, studies have yielded somewhat conflicting results as downregulation of PKC activity has been observed in cancerous tissue of chemically induced rat colon carcinogenesis (47,48) and human colonic carcinomas (49,50), while others have found no differences (51). It is now recognized that PKC may translocate to the nucleus (52) and thus participate in biological responses.

In the present investigation, PKC activity in the cytosolic and particulate fractions was measured using a method similar to those employed by others for studying colonic epithelium (13,16,43,46,53). Considerable advances have been made in the biochemical and molecular approaches to study various PKC isoforms and their role in cellular processes. In our view, the observation that PKC activity was modulated in the normal mucosa by dietary fat and that the level of various PKC isoforms may differ in normal vs. neoplastic colonic tissue (53,54) does lend support to the notion that PKC plays an important role in colon carcinogenesis. How a tumor-promoting environment affects PKC activity and its isoforms in normal and neoplastic tissue remains to be seen and should be explored in future studies.

The ability of the high-fat diet to increase the PC/PE ratio in the colonic mucosa is similar to findings reported for the colonic mucosa of mice fed a high corn oil or beef tallow diet (55). Although the initial activation of PKC is dependent on the hydrolysis of phosphatidylinositol, Nishizuka (11) proposed that this transient stimulation is frequently followed by a more sustained increase in DAG derived through the hydrolysis of PC. It is, therefore, plausible that the increased PKC activity observed in the high-fat fed animals may in part be attributable to an increased PC/PE colonic mucosal ratio. Whether it is the increased PC/PE ratio or the decreased PE content of the cellular membranes which affects PKC directly still remains to be established. The consistent increase in the percentage of 20:4n-6 in response to the high-fat diet in both the PC and PE fractions is worth noting and may be of biological importance. Arachidonic acid and its metabolites have been associated with neoplastic events and have been proposed to affect signal transduction by affecting the PKC activation pathway (55).

In summary, our results demonstrate that a high-fat diet rich in saturated fatty acids markedly affects the preneoplastic state of the colonic epithelium. Moreover, although this diet exerted minor effects on the lipid composition of the tissue, it exerted a distinct modulating effect on the activity of PKC, an enzyme critically associated with cell growth and differentiation and the carcinogenic process.

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Linoleic Acid Uptake by Isolated Enterocytes: Influence of α-Linolenic Acid on Absorption

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In a previous study we showed that intestinal uptake of α-linolenic acid (18:3n-3) was carrier-mediated and we suggested that a plasma membrane fatty acid protein was involved in the transport of long-chain fatty acids. To further test this hypothesis, the mechanism of linoleic acid (18:2n-6) uptake by isolated intestinal cells was examined using a rapid filtration method and 20 mM sodium taurocholate as solubilizing agent. Under these experimental conditions transport of [1-^{14}C]linoleic acid monomers in the concentration range of 2 to 2220 nM was saturable with a V_m of 5.1 ± 0.6 nmol/mg protein/min and a K_m of 183 ± 7 nM. Experiments carried out in the presence of metabolic inhibitors, such as 2,4-dinitrophenol and antimycin A, suggested that an active, carrier-mediated mechanism was involved in the intestinal uptake of this essential fatty acid. The addition of excess unlabeled linoleic acid to the incubation medium led to a 89% decrease in the uptake of [1-^{14}C]linoleic acid, while D-glucose did not compete for transport into the cell. Other long-chain polyunsaturated fatty acids added to the incubation mixture inhibited linoleic acid uptake by more than 80%. The presence of α-linolenic acid (18:3n-3) in the incubation medium caused the competitive inhibition (K_i = 353 nM) of linoleic acid uptake. The data are compatible with the hypothesis that intestinal uptake of both linoleic, and α-linolenic acid is mediated by a membrane carrier common to long-chain fatty acids.

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Linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) are essential fatty acids that share the same desaturation and chain elongation pathways (1–3) and produce metabolites that are incorporated into membrane phospholipids and body tissues (4). The two fatty acids compete with each other particularly at the limiting Δ6 desaturase step (1–3). Several authors have investigated the effects of the n-6/n-3 fatty acid ratio in the diet with focus on the metabolic steps leading to the synthesis of eicosanoids (5–9). On the other hand, few studies have addressed the possibility that high levels of 18:2n-6 in the diet may interfere with 18:3n-3 uptake by intestinal cells, and *vice versa* (10,11). There is increasing evidence showing that cellular uptake of long-chain fatty acids occurs by a process of facilitated membrane translocation involving a membrane-bound fatty acid binding protein (12–14).

In a previous study we observed that α-linolenic acid uptake by intestinal cells was carrier-mediated and that addition of linoleic acid to the incubation medium led to a decrease in the initial rate of α-linolenic acid uptake (15). Based on these observations, we hypothesized that the parent essential fatty acids were transported by the same transport protein. This hypothesis could be questioned in the light of the facilitated (16–18) or passive (19) diffusion mechanism of linoleic acid across membrane enterocytes reported by others. Indeed, the mechanism of α-linolenic acid uptake that we observed in the presence of linoleic acid could be related to an allosteric inhibition of the transport protein without any concomitant uptake of linoleic acid *via* the same carrier. In order to address this point, it was necessary (i) to determine the kinetic parameters of the initial rate of uptake of linoleic acid and (ii) to examine whether the intestinal uptake of these essential fatty acids is linked by a competitive or noncompetitive inhibition mechanism.

In the present study we examined the kinetics of linoleic acid uptake by isolated hamster intestinal cells using sodium taurocholate as solubilizing agent. The effect of other long-chain fatty acids, in particular α-linolenic acid, on linoleic acid uptake was also determined. The results show that linoleic acid uptake is active and carrier-mediated and that α-linolenic acid added to the incubation medium leads to the competitive inhibition of linoleic acid uptake by intestinal cells.

MATERIALS AND METHODS

Chemicals and solutions. Taurocholic acid sodium salt, cholesterol, D-glucose, antimycin A, 2,4-dinitrophenol, collagenase type IA, bovine serum albumin essentially fatty acid free, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), Hanks' medium, free fatty acids (linoleic, α-linolenic, γ-linolenic, linolelaidic, arachidonic, eicosatrienoic, eicosapentaenoic, docosatetraenoic), linoleic anhydride and linolenic acid methyl ester were obtained from Sigma Chimie (St. Quentin Fallavier, France). [1-^{14}C]Linoleic acid (1.9 GBq/mmol) was purchased from New England Nuclear (DuPont de Nemours, Paris, France). The scintillation liquid Optiphase 'Hisafe' II was from LKB (Pharmacia, St. Quentin en Yvelines, France). Solutions of labeled linoleic acid were prepared by isotopic dilution. For the uptake studies, the fatty acids and all other reagents were solubilized at room temperature in Hanks' medium containing 20 mM taurocholate by sonication for 5 min in a Ney 300 water-bath sonicator (Bioblock Scientific, Illkirch, France).

Preparation of intestinal cells. Intestinal cells were isolated as previously described (15,20). Briefly, male hamsters weighing 100–120 g (Elevage Dépré, Saint Doulchard, France) were killed by overexposure to diethyl ether. The entire small intestine was removed, rinsed with oxygenated buffer solution containing sodium citrate and incubated for 10 min in the same buffer at 37°C. The intestine was then emptied, filled with oxygenated buffer solution containing ethylenediaminetetraacetic acid, incubated for 3 min at 37°C, and then gently palpated with

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

the fingers for 2 min. This treatment was repeated twice. The buffer containing intestinal cells was recovered in Hanks' solution. After centrifugation ($765 \times g$), the isolated cells were resuspended in Hanks' medium containing collagenase type IA (37°C, 15 min with stirring). The intestinal cells were then washed twice and resuspended in Hanks' medium (cellular stock solution containing about 5 mg protein/mL). Light microscopy of the suspension showed that about 90% of the cells displayed the typical features of villus-tip cells. Viability was assessed by trypan blue exclusion and by lactate dehydrogenase release assay using the lactate dehydrogenase (EC 1.1.1.27) optimized kit DG 1340-K (Sigma Chimie). Protein concentration was assayed by the method of Lowry *et al.* (21).

Determination of linoleic acid monomer concentration. Ultrafiltration and polyethylene partitioning were used to measure monomer concentrations. (i) Ultrafiltration method: Linoleic acid monomers in the micellar solution were determined by ultrafiltration through a 1,000-molecular weight cut-off membrane YM1 (Grace SA, Amicon Division, Epernon, France) as described previously (15). Radioactivity was determined in the initial micellar solution, the filtrate, and in the retained solution after ultrafiltration. The recovery of the radioactivity initially present in the micellar solution was found to be complete, thus no adsorption occurred on the ultrafiltration membrane. Linoleic acid molar concentration was calculated from the specific activity of [1-^{14}C]linoleic acid. The percentage of monomer fatty acid in the micellar solution was calculated as the ratio of concentration in the filtrate against the concentration in the retained fraction. (ii) Polyethylene partitioning system: This technique is based upon the partitioning of fatty acids between the aqueous solution and a solid organic phase, namely a polyethylene disc. Using this method, Sallee (22) demonstrated that monomer activities of solutions of long-chain fatty acids dissolved in 20 mM bile acid were linearly dependent on the total fatty acid concentration in the incubation medium. Polyethylene discs, 1.2 cm in diameter, were punched from polyethylene film, 0.16 mm thick, manufactured by Roth Sochiel (Lauterbourg, France). To remove any oil and debris, the discs were washed in methanol and distilled water and were dried before use. After equilibration of two discs in 3 mL of fatty acid test solutions for 24 h (shaking waterbath, 37°C), the discs were removed, rinsed in Hanks' medium and the radioactivity associated with the discs was determined.

Determination of linoleic acid uptake. Uptake of [1-^{14}C]linoleic acid was measured by the rapid vacuum filtration assay. One mL of cellular stock suspension was incubated for 1 min in a 37°C waterbath with stirring, and uptake was started by the addition of 1 mL of fatty acid solution (Hanks' medium plus 20 mmol/L sodium taurocholate) containing [1-^{14}C]linoleic acid at 37°C. At various time points, 200-μL sample aliquots (about 0.5 mg protein) were pipetted into 3 mL of 5 mg albumin/mL Hanks' medium (4°C) to stop cellular influx and to remove bound fatty acids. In separate experiments it was found that resuspending the cells in 5 mg albumin/mL at 4°C stopped uptake after a rapid reequilibration period of 1 min. During this time about 10% of cell-associated radioactivity was removed, and no further change in radioactivity was detected during the next 10 min. The stop solution containing cells was pipetted onto the center of Whatman GF/C glass microfibre filter (Poly Labo, Strasbourg, France), and filtered under 8×10^4 P_a vacuum pressure using a filtration apparatus (Hoefer model FH225V; Bioblock). Cells were washed with 5 mL of 5 mg albumin/mL solution (4°C) and thereafter with 20 mL of Hanks' medium (4°C). Filters were placed in scintillation vials, 4 mL of scintillation liquid were added and radioactivity was determined in a LKB-1215 Rackbeta liquid scintillation counter (Pharmacia). Nonspecific radioactivity binding to filters and cells was measured in each experiment by adding 5 mg albumin/mL Hanks' medium (4°C) before the addition of corresponding aliquots of cells and [1-^{14}C]-linolenic acid working solutions. The value of each sample was the net radioactivity after subtraction of this blank.

The effect of the metabolic inhibitor antimycin A or 2,4-dinitrophenol (final concentrations 25 and 250 μM, respectively) was studied by adding the inhibitor to the cells 10 min before linoleic acid solubilized in taurocholate was added. In studies on the effect of Na^+ depletion, NaCl was replaced by isoosmotic choline chloride. Cells were first washed twice in the Na^+ depleted medium and then incubated for 10 min at 37°C in the same medium. One mL of [1-^{14}C]linoleic acid was then added to 1 mL of cell suspension (final concentration 100 μM linoleic acid in 20 mM taurocholate), and uptake was determined as described above. The experimental protocol was changed somewhat in studies on the effect of other unlabeled substrates (100 μM or 1 mM final concentration) on linoleic acid uptake. Nine hundred μL of unlabeled substrate solubilized in 20 mM taurocholate was added to 1 mL of cell suspension. After 30 s at 37°C with stirring, 100 μL of [1-^{14}C]-linoleic acid was added (final concentration 100 μM in 20 mM taurocholate), and the initial rate of uptake was determined as described above.

Statistical analysis. Initial rates of uptake were calculated by the least-squares method which allows an estimation of simple linear regression parameters and their standard deviations. Comparisons of ordinates at origin with zero value were evaluated by Student *t*-test. Curves characterizing the intracellular uptake of linoleic acid were calculated with Microsoft® Excel solver (Les Ulis, France) and P.C.S.M. program (Deltasoft, Grenoble, France). The program provides the kinetic parameters (V_m, K_m) of a nonlinear function (Michaelis-Menten equation) with the least-squares method. Comparisons between group means were evaluated by unpaired *t*-test. Statistical significance of differences among more than two groups were determined by analysis of variance.

RESULTS

Determination of the concentration of linoleic acid monomers in the incubation medium. Linoleic acid transport by intestinal cells was studied in the concentration range of 1 to 1000 μM. When the fatty acid was solubilized in the incubation medium containing a bile salt (sodium

taurocholate), a mixture of mixed micelles of fatty acid/taurocholate and free fatty acids (monomer) was obtained. The micelles cannot readily pass through cell membranes due to their higher molecular weight, and it is generally accepted that only fatty acids present as monomers can participate in the absorption process (23). The membrane ultrafiltration method we used made it possible to determine the quantity of monomeric fatty acid in the solution studied (1 to 1000 μM linoleic acid in 20 mM sodium taurocholate). Figure 1 shows that the monomer concentration was proportional to the quantity of linoleic acid present in the incubation medium. The Y-intercept of this line was not significantly different from zero, and the partition coefficient between fatty acids in the extra- and intramicellar phase was constant ($K = 2.22 \times 10^{-3}$).

Another estimation of the monomer activities was based on the polyethylene disc method (see Materials and Methods section). Figure 2 shows the relationship between the linoleic acid concentration in the incubation medium and the quantity of fatty acid taken up by the polyethylene discs. The relationship between these two parameters was linear as long as the linoleic acid concentration in the incubation medium was lower than 400 μM.

Kinetics of intracellular uptake of linoleic acid. Figure 3 is an example of uptake kinetics by isolated intestinal cells of linoleic acid at 200 μM solubilized in 20 mM taurocholate. Uptake was maximal and linear during the first 20 s of incubation, and then decreased progressively. The initial uptake rate of the fatty acid (V_0) corresponds to the slope of the line obtained by fitting the experimental values measured during the first 20 s of incubation to a linear model. The example of Figure 3 gives $V_0 = 3.57$ nmol/mg protein/min (mean $V_0 = 3.67 \pm 0.57$ nmol/mg protein/min).

A reduction in linoleic acid transport of more than 80% was observed when the temperature of the incubation medium was decreased from 37 to 4°C (Fig. 3). A lower level of uptake was also noted when intestinal cells were incubated for 10 min at 37°C in the presence of metabolic inhibitors. In comparison to controls, 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (250 μM), and antimycin A, an inhibitor of the mitochondrial electron transport system (25 μM), caused a considerable decrease in the initial rate of uptake of 100 μM linoleic acid (2.18 ± 0.22, 0.66 ± 0.21, 0.32 ± 0.20 nmol/mg protein/min, respectively). This drop in fatty acid uptake was much greater than the decrease in intestinal cell viability seen due to these metabolic inhibitors (<7%).

Influence of linoleic acid concentration on initial rate of uptake. The initial rates of fatty acid uptake were measured for different concentrations (1 to 1000 μM) of linoleic acid solubilized in 20 mM taurocholate. When initial rates were plotted as a function of monomer concentration as

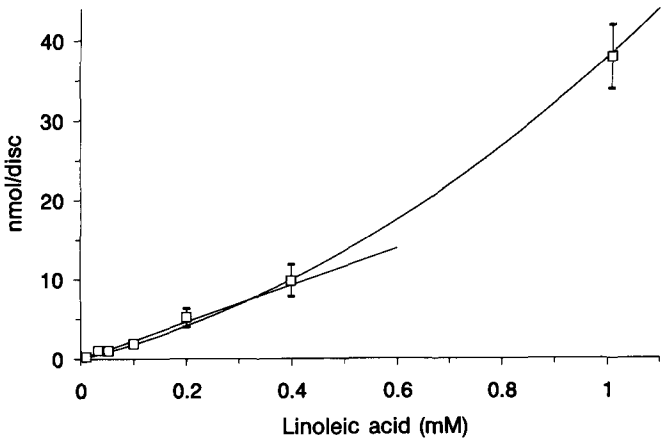

FIG. 2. Characteristics of partitioning of linoleic acid into polyethylene discs and apparent monomeric activity of linoleic acid in 20 mM taurocholate micellar solutions. Values are means ± SE of four experiments. Absent error bars indicate that errors fell within symbol.

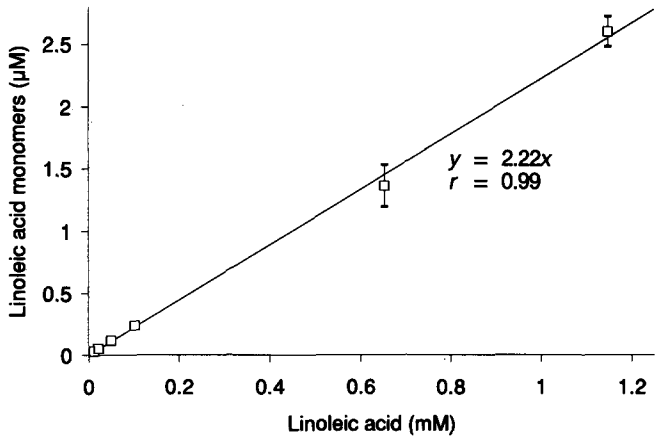

FIG. 1. Effect of the level of linoleic acid solubilized in 20 mM taurocholate on linoleic acid monomer concentration. Monomers were separated from micellar aggregates by ultrafiltration as described in the Materials and Methods section. Values are means ± SE of three experiments. Absent error bars indicate that errors fell within symbol.

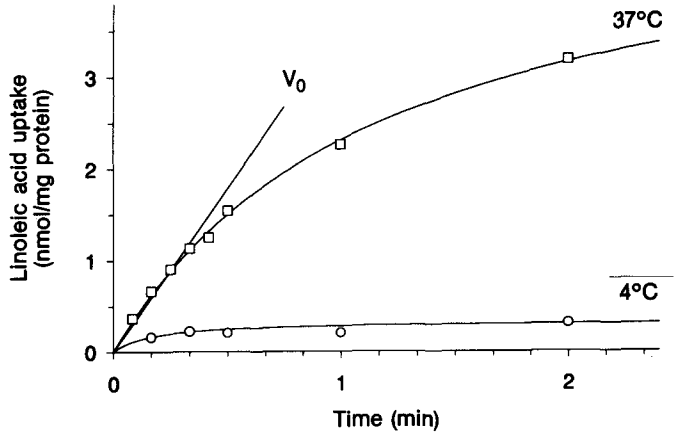

FIG. 3. Time course of linoleic acid uptake by isolated enterocytes at 37 and 4°C. Linoleic acid (200 μM) was solubilized in 20 mM taurocholate. The initial uptake rate (V_0) was calculated by the least-squares method from the uptake obtained over the initial 20-s incubation period. Values are means ± SE of four experiments. All error bars fell within the symbols.

determined by ultrafiltration (2 to 2220 nM), saturation of uptake was observed (Fig. 4). When the experimental values were fit to the Michaelis-Menten model, V_m = 5.1 ± 0.6 nmol/mg protein/min and K_m = 183 ± 7 nM were calculated.

Effect of long-chain fatty acids and other substrates on the uptake of linoleic acid. In order to determine whether the uptake of linoleic acid by intestinal cells was modified by the presence of other substrates in the incubation medium, long-chain polyunsaturated fatty acids, certain derivatives of linoleic acid, or D-glucose, each at 1 mM, were added to the micellar solution containing 100 μM [1-^{14}C]linoleic acid and 20 mM taurocholate. The addition of long-chain fatty acids reduced the uptake of linoleic acid by more than 80% (Table 1). This inhibition was observed for fatty acids with different chain lengths or different degrees of unsaturation. On the other hand, changes in the uptake of [1-^{14}C]linoleic acid induced by linoleic acid analogues were dependent on the type of derivative used. When the terminal carboxyl group of linoleic acid was blocked (linoleic acid methyl ester, linoleic anhydride), the inhibition of uptake was attenuated. Finally, when the incubation medium was supplemented with a molecule that is actively transported by intestinal cells, but is unrelated to a fatty acid (D-glucose), there was no change in linoleic acid uptake. The inhibition of linoleic acid influx observed in the presence of unlabeled fatty acids was not related to a decrease in the monomer concentration of [1-^{14}C]linoleic acid in the incubation medium. The polyethylene disc technique indicated no statistically significant difference between linoleic acid monomers present in the incubation medium before and after the addition of 1 mM unlabeled substrate (Table 2).

Effect of α-linolenic acid on the uptake of linoleic acid. The initial rate of [1-^{14}C]linoleic acid uptake (50 to 500 μM solubilized in 20 mM taurocholate) was measured with and without the addition of a constant quantity of α-linolenic acid (100 μM solubilized in 20 mM taurocholate) to the incubation medium. A Lineweaver-Burk plot of the data (Fig. 5) shows that the addition of α-linolenic acid to the incubation medium changed the slope that characterizes [1-^{14}C]linoleic acid uptake (K_m/V_m), but had no significant effect on the Y-intercept ($1/V_m$). In other words, addition of α-linolenic acid caused a change in K_m without modifying V_m for [1-^{14}C]linoleic acid. The decreased uptake observed coincided with competitive inhibition, with an apparent K_i of 157 μM, corresponding to an α-linolenic acid monomer concentration of 353 nM.

Effect of extracellular sodium on linoleic acid uptake. The effect of extracellular sodium on the uptake of 100 μM linoleic acid dissolved in 20 mM taurocholate was tested by replacing the sodium chloride in the incubation medium by an isoosmotic concentration of choline chlo-

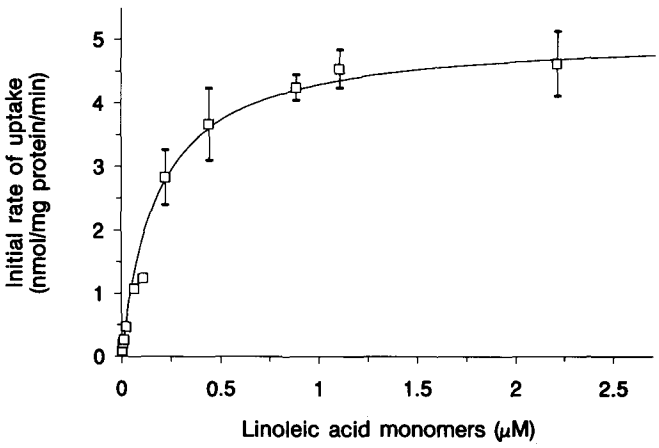

FIG. 4. Relationship between linoleic acid monomer concentration in the incubation medium and initial uptake rate. Values are means ± SE of four separate experiments. Kinetics parameters were generated from weighted least-square fits of the individual data points from each experiment to a rectangular hyperbola (V_m = 5.1 ± 0.6 nmol/mg protein/min; K_m = 183 ± 7 nM).

TABLE 1

Effect of Addition of Various Unlabeled Substrates on Cellular Uptake of 100 μM [1-^{14}C]Linoleic Acid Solubilized in 20 mM Taurocholate[a]

Additive[b]	Initial rate of uptake[c]
None (control)	2.61 ± 0.15
Fatty acids	
Linoleic acid (18:2n-6)	0.29 ± 0.05[e]
Linolelaidic acid (*trans*-18:2n-6)	0.15 ± 0.05[e]
α-Linolenic acid (18:3n-3)	0.24 ± 0.03[e]
γ-Linolenic acid (18:3n-6)	0.50 ± 0.06[e]
all-cis-8,11,14-Eicosatrienoic acid (20:3n-6)	0.23 ± 0.08[e]
Arachidonic acid (20:4n-6)	0.17 ± 0.06[e]
Eicosapentaenoic acid (20:5n-3)	0.21 ± 0.04[e]
Docosatetraenoic acid (22:4n-6)	0.42 ± 0.03[e]
Others	
Linoleic acid methyl ester	1.80 ± 0.07[d]
Linoleic anhydride	2.04 ± 0.11[d]
D-Glucose	2.46 ± 0.08

[a]Results are given as means ± SE of four experiments.
[b]Final concentration of additive in the incubation medium: 1 mM.
[c]Initial rate of uptake is expressed as nmol/mg protein/min. Comparison to control [d]$P < 0.05$, [e]$P < 0.001$.

TABLE 2

Effect of Various Unlabeled Substrates on Linoleic Acid Content of Polyethylene Discs[a]

Additive[b]	Linoleic acid per disc[c]
None (control)	1717 ± 53
Fatty acids	
Linoleic acid (18:2n-6)	1908 ± 24
α-Linolenic acid (18:3n-3)	1913 ± 69
Arachidonic acid (20:4n-6)	2035 ± 70
Others	
Linoleic acid methyl ester	1542 ± 58
D-Glucose	1764 ± 30

[a]Incubation medium contained 100 μM linoleic acid solubilized in 20 mM taurocholate. Results are given as means ± SE of four experiments.
[b]Final concentration of additive in the incubation medium: 1 mM.
[c]Linoleic acid content of polyethylene disc is expressed as pmol/disc. There is no statistical difference ($P > 0.05$) between the means (analysis of variance).

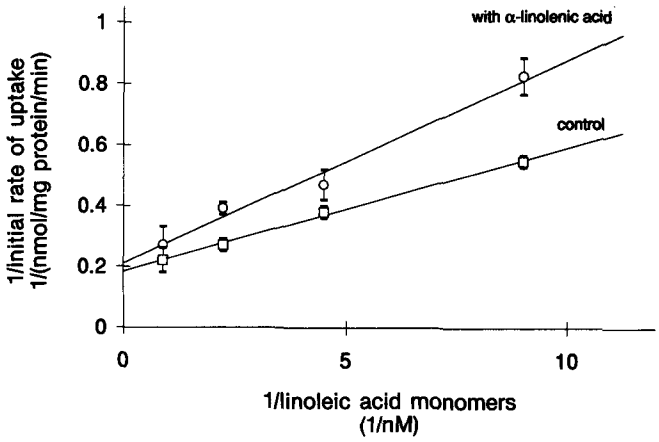

FIG. 5. Lineweaver-Burk plot of cellular [1-^{14}C]linoleic acid uptake in the presence (circles, Y = 0.2097 + 0.0303 X, r = 0.973) and absence (squares, Y = 0.1839 + 0.0185 X, r = 0.984) of 100 μM α-linolenic acid in the incubation medium. The addition of α-linolenic acid changed the K_m value without changing the V_m value of linoleic acid uptake, revealing a predominantly competitive type of inhibition by α-linolenic acid. Values are means ± SE of four experiments.

ride. Nevertheless, the presence of sodium taurocholate as solubilizing agent requires a minimum of 20 mM Na^+ in the incubation medium. In Na^+-depleted medium, uptake of linoleic acid was reduced (1.05 ± 0.07 nmol/mg protein/min) compared with the presence of Na^+ (1.82 ± 0.06 nmol/mg protein/min, this control value was lower than usually seen due to additional washes used in this protocol). The decreased uptake was associated with a decrease in monomer activity of linoleic acid in the incubation medium (839 ± 16 pmol/disc in the Na^+-depleted medium and 1940 ± 74 pmol/disc in presence of Na^+) probably because of changes in the ionic strength in the incubation medium. Taking into account this decrease in monomer concentration, we arrived at an initial rate of uptake of 0.99 nmol/mg protein/min. This calculated value for V_i was quite similar to that reported for Na^+-depleted medium. Thus, a direct relationship between Na^+-depleted medium and reduced linoleic acid uptake seems unlikely.

DISCUSSION

Prior work in our laboratory had shown that the uptake of α-linolenic acid (18:3n-3) by isolated hamster intestinal cells was carrier-mediated (15). This was concluded based on results obtained utilizing rapid filtration through glass microfiber filter with a bile salt present as solubilizing agent. Appropriate dispersion of fatty acids in the incubation medium is indispensable when studying fatty acid absorption by intestinal cells. Several authors have used bovine serum albumin to complex fatty acids, and the proportion of unbound fatty acid (monomers) in the incubation medium could thus be modulated by varying the fatty acid/albumin ratio (11,24,25). This approach is thought to be physiologically appropriate for cells that are naturally in contact with circulating plasma, e.g., liver cells. In intestinal absorption, however, fatty acids are solubilized by bile salts, and to mimic physiological conditions, the fatty acids used in this study were solubilized by the addition of 20 mM sodium taurocholate. We have shown that sodium taurocholate had no effect on the viability or permeability of intestinal cells when the incubation time was shorter than 1 min (15), as used in the present study. Membrane ultrafiltration (1,000 molecular weight cut-off) showed that the monomer concentration increased proportionally to the total concentration of linoleic acid solubilized in the incubation medium (Hanks' medium plus 20 mM sodium taurocholate). The partition coefficient between monomers and micelles observed for linoleic acid ($K = 2.22 \times 10^{-3}$) was similar to that found for α-linolenic acid ($K = 2.25 \times 10^{-3}$) (15).

The results obtained in the present study indicate that linoleic acid uptake by isolated hamster intestinal cells depends on an active and carrier-mediated mechanism. Several points argue in favor of this type of transport:

(i) The initial rate of linoleic acid uptake was not proportional to the monomer concentration in the incubation medium. The initial rate of fatty acid uptake reached a plateau when the monomer concentration was higher than 1000 nM. Kinetics were saturable, and the Michaelis-Menten model led to the calculation of V_m = 5.1 ± 0.6 nmol/mg protein/min and K_m = 183 ± 7 nM. This saturable transport mechanism excludes the simple diffusion mechanism proposed for this fatty acid by Ling *et al.* (19) and agrees with results obtained *in vitro* (16) and *in vivo* (17) by Chow and Hollander and by Molina *et al.* (18) using everted intestinal sacs. With respect to intestinal uptake, the data obtained for oleate (13), α-linolenate (15) and linoleate (this study), strongly support the carrier-mediated transport of C18 fatty acids.

(ii) Lowering the temperature of the incubation medium from 37 to 4°C caused a considerable reduction in the initial rate of linoleic acid uptake. This may in part be due to a decrease in monomer concentration. However, the action of metabolic inhibitors, such as 2,4-dinitrophenol and antimycin A, caused a considerable drop in fatty acid uptake. This suggests that the process of linoleic acid uptake by intestinal cells requires cellular energy. The uptake of linoleic acid, as that of α-linolenic acid, is thus dependent on active transport. With respect to the role of energy in linoleic acid transport, our results disagree with work published by others (16–18). As we mentioned earlier (15), however, the previous authors (16–18) studied the absorption of linoleic acid using models and experimental conditions that did not permit them to assess the effect of temperature and metabolic inhibitors on the initial rate of fatty acid uptake.

(iii) Involvement of an active, carrier-mediated mechanism presupposes the existence of a transport protein. In order to clarify this point, we determined the effect of several polyunsaturated fatty acids, including linoleic acid itself, on the uptake of labeled linoleic acid. The separate addition of fatty acids to the incubation medium resulted in a significant decrease in the initial rate of linoleic acid uptake. This considerable decrease in linoleic acid uptake was apparently not related to the type of fatty acid added in large excess to the incubation medium. The terminal carboxyl group of the fatty acid, on the other hand, played

an important role in the recognition of linoleic acid by the transporter.

The decreased uptake of linoleic acid we observed could nevertheless be related to a disturbance in the monomer/micelle balance induced by addition of a high concentration of fatty acids to the incubation medium (16,26). Although this cannot be ruled out, it seems improbable since the monomer concentration of linoleic acid remained unchanged after the addition of different substrates, based on data obtained by the polyethylene disc method.

Both the active and carrier-mediated uptake of linoleic acid, and the inhibitory effect of other fatty acids, argue in favor of the presence of a membrane transport protein that can be shared by several long-chain fatty acids. In our previous work (15) the existence of this common carrier was stipulated based on the inhibition of α-linolenic acid uptake induced by large amounts of several polyunsaturated fatty acids, particularly linoleic acid. It was not clear, however, whether the inhibition observed was competitive or noncompetitive. In the present study, this point was examined for the parent essential fatty acids, α-linolenic and linoleic acids. The addition of α-linolenic acid to micellar solutions of linoleic acid changed the K_m characterizing the uptake without significantly changing V_m. When α-linolenic acid was added to the incubation medium, a competitive inhibition of linoleic acid uptake was seen. The inhibition constant we calculated (K_i = 353 nM) is very close to the K_m we reported for α-linolenic acid (15).

Our results suggest that uptake of the two essential fatty acids, linoleic and α-linolenic acids, by intestinal cells is brought about by the same transport protein, probably the 40 kDa plasma membrane fatty acid binding protein described by Stremmel *et al.* (12) to occur in intestinal cells (12) and which was subsequently also found in other tissues (27). Within the C_{18} fatty acids, the K_m differs for different fatty acids. A K_m of 93 nM was reported for oleic acid (13), of 183 nM for linoleic acid (present study) and of 382 nM for α-linolenic acid (15). These differences suggest that for fatty acids of the same carbon-chain length, the number and/or the position of double-bounds can modulate the affinity to the common membrane transport protein.

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Occurrence of 5*c*,8*c*,11*c*,15*t*-Eicosatetraenoic Acid and Other Unusual Polyunsaturated Fatty Acids in Rats Fed Partially Hydrogenated Canola Oil

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Uncommon *cis* and *trans* fatty acids can be desaturated and elongated to produce unusual C_{18} and C_{20} polyunsaturated fatty acids in animal tissues. In the present study we examined the formation of such metabolites derived from *cis* and *trans* isomers of oleic and linoleic acids of partially hydrogenated vegetable oil origin in rats. For two months, adult male rats were fed a partially hydrogenated canola oil diet containing moderately high levels of *trans* fatty acids (9.6 energy%) and an adequate level of linoleic acid (1.46 energy%). Analysis of the phospholipid (PL) fatty acids of liver, heart, serum and brain showed no new C_{18} polyunsaturated fatty acids, except for those uncommon 18:2 isomers originating from the diet. However, minor levels (each <0.3% PL fatty acids) of six unusual C_{20} polyunsaturated fatty acids were detected in the tissues examined, except in brain PL. Identification of their structures indicated that the dietary 9*c*,13*t*-18:2 isomer, which is the major *trans* polyunsaturated fatty acid in partially hydrogenated vegetable oils, was desaturated and elongated to 5*c*,8*c*,11*c*,15*t*-20:4, possibly by the same pathway that is operative for linoleic acid. Furthermore, dietary 12*c*-18:1 was converted to 8*c*,14*c*-20:2 and 5*c*,8*c*,14*c*-20:3; dietary 9*c*,12*t*-18:2 metabolized to 11*c*,14*t*-20:2 and 5*c*,8*c*,11*c*14*t*-20:4, and dietary 9*t*,12*c* to 11*t*,14*c*-20:2. These results suggested that of all the possible isomers of oleic and linoleic acids in partially hydrogenated vegetable oils, 12*c*-18:1, 9*c*,13*t*-18:2, 9*c*,12*t*-18:2 and 9*t*,12*c*-18:2 are the preferred substrates for desaturation and elongation in rats. However, their conversions to C_{20} metabolites were not as efficient as that of oleic or linoleic acids.

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Partially hydrogenated vegetable oils (PHVO) contain a range of *cis* and *trans* isomers of oleic (9*c*-18:1) and linoleic (9*c*,12*c*-18:2) acids (1–5). Studies with rodents have shown that some of these isomers are desaturated and elongated to unusual C_{18} and C_{20} polyunsaturated fatty acids (PUFA) (6–8).

Cis-9,*trans*-13-octadecadienoic acid (9*c*,13*t*-18:2) is the major *trans* polyunsaturated isomer in PHVO (2,5,9). During gas chromatographic analysis, this isomer co-elutes with 8*t*,12*c*-18:2, which is a very minor component in PHVO (2). Therefore, the amounts of these two isomers are generally listed together in reports giving the fatty acid composition of dietary fats. Canadian margarines contain 0.5–2.6% of the two isomers (2), and many bakery products contain similar levels (10). Levels as high as 9% were reported for some partially hydrogenated soybean oil stocks (5). Information on the metabolism of the two mono-*trans* geometrical isomers of linoleic acid, 9*c*,12*t*-18:2 and 9*t*,12*c*-18:2 (5–7,11,12), which are also present in appreciable quantities in PHVO, is available, but nothing is known about the metabolism of 9*c*,13*t*-18:2. The Δ6 desaturase is active only on fatty acids with a *cis*-9 double bond, and, hence, it may be expected that the 9*c*,13*t*-18:2 is converted to a *t*-20:4 isomer, similar to the conversion of 9*c*,12*t*-18:2 to a *trans* isomer of arachidonic acid in rodents (5–7,12).

The present study, therefore, examined the possible formation of PUFA metabolites of 9*c*,13*t*-18:2 in rats. Because 9*c*,13*t*-18:2 is not commercially available, a mildly hydrogenated canola oil, with 9*c*,13*t*-18:2 + 8*t*,12*c*-18:2 content of 1.9%, was used as the dietary source of this acid. In addition, we report the occurrence of other unusual C_{20} PUFA and the compositions of 18:2 and 18:1 isomers in the rat. The fatty acid data were compared to a reference group of rats fed an unhydrogenated canola oil diet.

MATERIALS AND METHODS

Fatty acid methyl esters (FAME) standards were purchased from Sigma Chemical Company (St. Louis, MO). Several unsaturated FAME required for the identification of unusual C_{18} and C_{20} PUFA were prepared from 9*c*,12*c*-18:2, 9*c*,12*c*,15*c*-18:3, 11*c*,14*c*-20:2, 8*c*,11*c*,14*c*-20:3 and 5*c*,8*c*,11*c*,14*c*-20:4 standards, through partial reduction with hydrazine, geometrical isomerization with *p*-toluenesulfinic acid and fractionation of the products by $AgNO_3$–thin-layer chromatography (TLC) (2,13–15).

The partially hydrogenated canola oil (PHCO) (iodine value 94) used in the rat diet was supplied by Canamara Foods (Toronto, Ontario, Canada). Unhydrogenated canola oil was purchased from a supermarket in Ottawa.

Animals and diets. Male Sprague-Dawley rats (Charles River, Quebec, Canada), weighing 244 ± 3 g, were housed individually in metal cages in an air-conditioned room maintained at 22°C and 60% relative humidity with a 12-h day/12-h night cycle. The rats were randomly assigned to two groups of ten rats per group. The animals were fed isocaloric (468 Kcal/100 g diet), semisynthetic diets containing either 20% PHCO or 20% canola oil. The other ingredients in the diet were, in g/100 g diet, vitamin-free ca-

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Abbreviations: DMOX, 2-alkenyl-4,4-dimethyloxazoline; ECL, equivalent chain length; en%, energy%; EFA, essential fatty acids; FAME, fatty acid methyl esters; GC, gas chromatography; HPLC, high-pressure liquid chromatography; GC/MS, GC/mass spectrometry; MI, methylene interrupted; NMI, nonmethylene interrupted; PL, phospholipids; PHCO, partially hydrogenated canola oil; PHSO, partially hydrogenated soybean oil; PHVO, partially hydrogenated vegetable oil(s); TLC, thin-layer chromatography.

sein, 22.0; cornstarch, 27.0; sucrose, 22.8; alphacel, 3.0; mineral mixture-AIN 76, 3.5; vitamin mixture-AIN 76A, 1.0; choline bitartrate, 0.4; and DL-methionine, 0.3 (Teklad, Madison, WI). The diets were prepared weekly and stored at −4°C. Food and water were available to the animals *ad libitum*. The animals were killed after 60 d of feeding; liver, heart, brain and blood (aorta) were collected and immediately frozen in liquid nitrogen and stored at −80°C under nitrogen until analyzed for fatty acids.

Analytical procedures. Total lipids from the tissues were extracted with chloroform/methanol (2:1, vol/vol) (16). Phospholipids (PL) were isolated by TLC with development in hexane/diethyl ether/acetic acid (80:20:1, by vol). FAME of the PL and dietary oils were obtained following treatment with BF_3/MeOH (Sigma Chemical Company).

The FAME from liver PL of the ten rats fed the PHCO diet were pooled for determination of the 18:1 isomer composition and for characterization of the unusual C_{18} and C_{20} PUFA. The FAME were initially fractionated by semi-preparative high-pressure liquid chromatography (HPLC) on a Waters (Milford, MA) Bondapak C_{18} column using MeOH/H_2O (95:5, vol/vol) as mobile phase. Each HPLC fraction (four in all) was further fractionated by preparative $AgNO_3$–TLC with development either in toluene at −25°C (for separation of the fractions containing three or less double bonds) or hexane/diethyl ether (2:3, vol/vol) at room temperature (for separation of fractions containing four or more double bonds). This produced various fractions enriched with 18:1*c*, 18:1*t*, 18:2*tt*, 18:2*ct*/*tc* (methylene interrupted, MI) 18:2*ct*/*tc* (nonmethylene interrupted, NMI), 18:2*cc* (MI), 18:2*cc* (NMI), 20:2*ct*/*tc* (MI), 20:2*cc* (MI), 20:3*ccc* (NMI) 20:3*ccc* (MI), 20:4 (all isomers) and 20:5 + 22:4 + 22:5 + 22:6. Each step of the isolation procedure was monitored by gas chromatography (GC). The HPLC and $AgNO_3$–TLC fractionations were repeated several times to obtain sufficient material for structural characterization.

For the determination of the composition of the 18:1 and 18:2 isomers in the dietary PHCO, the FAME were fractionated by $AgNO_3$–TLC with development in toluene at −25°C (2).

The isomer distribution of the isolated 18:1*c* and *t* fractions was determined through oxidative ozonolysis (17). The structures of the various 18:2 isomer fractions were identified from the previous work at this laboratory (2).

The structures of C_{20} PUFA were initially identified by comparison of their GC retention times with those of authentic standards. The structures of some isomers were confirmed through partial hydrazine reduction followed by identification of the resultant *cis*- and *trans*-monounsaturated FAME. Finally, the double bond positions of all the PUFA were confirmed by GC/mass spectrometry (GC/MS) analysis of their 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives (18–20).

Silica gel plates (Whatman K5, 250 μm layer, 20 cm × 20 cm; Whatman Laboratory Division, Clifton, NJ) were impregnated by dipping in a 10% solution of $AgNO_3$ in acetonitrile for 30 min. The bands were detected under ultraviolet light at 254 nm, after spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol, and scraped into glass centrifuge tubes. A 1% solution of NaCl in 90% MeOH was added until the silver-dichlorofluorescein complex disappeared. Water was added, and the methyl esters were extracted with hexane.

FAME were analyzed by GC using a 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 (Palo Alto, CA). The column was operated isothermally (180°C), or the oven temperature was programmed. After 10 min at an initial temperature of 160°C, the temperature was increased at a rate of 1°C per min to 210°C and held at the final temperature for 20 min. The injector and detector temperatures were 250°C. Hydrogen was used as the carrier gas at a pressure of 20 psi. The equivalent chain length (ECL) values of the unsaturated fatty acids were calculated according to the method described by Ackman (21).

For GC/MS analysis, the isolated fractions or the total FAME from the liver PL were saponified and acidified to the free acids and then converted to the DMOX derivative by treating with 2-amino-2-methylpropanol in a microvial at 170°C for 1 h, according to the procedure of Zhang *et al.* (18). Mass spectra of the DMOX derivatives were obtained on a VG Analytical MS system (Model 7070EQ; VG Analytical, Manchester, England), equipped with an 11/250 data system interfaced to a Varian GC (Model Vista 6000; Varian Associates, Palo Alto, CA) operated at an ionization energy of 70 eV. GC separation of the DMOX derivatives was performed on the same SP-2560 capillary column described for the FAME analysis. Helium was the carrier gas. The GC column oven temperature was programmed from 70 to 120°C at 50°C/min and from 120 to 220°C at 1.5°C/min.

RESULTS

Fatty acid composition of dietary fats. The fatty acid composition of the two diets is summarized in Table 1. Both diets contained comparable amounts of saturated fat as well as total *c*-18:1. However, the PHCO diet contained lower proportions of essential fatty acids (EFA) (3.8% vs. 30.3%) and higher proportions of *trans* fatty acids (26.6% vs. 0.2%). The *t*-18:1 isomers of PHCO ranged from Δ8 to Δ16, whereas the *c*-18:1 isomers ranged from Δ6 to Δ16. In contrast, unhydrogenated canola oil contained only two *c*-18:1 isomers, oleic (*c*-9) and *cis*-vaccenic (*c*-11), and there were no *t*-18:1 isomers.

The unusual 18:2 isomer content in PHCO was 7.6% of the total fatty acids. The 18:2 fraction consisted of several isomers, of which 9*c*,13*t*-18:2 was the major component. *trans*-PUFA were also present in the unhydrogenated canola oil, but at much lower levels, derived from the mono-*trans* geometrical isomers of linoleic and α-linolenic acids.

Food consumption, body and organ weights. Rats fed different diets consumed similar amounts of food (20:4 ± 3.1 g/rat/d), showed no sign of EFA deficiency and appeared healthy throughout the experimental period. The body, liver, heart and kidney weights of the PHCO group were 574.4 ± 34.9, 16.2 ± 2.9, 1.4 ± 0.2 and 3.1 ± 0.4 g, respectively, and did not differ from those of the reference group.

TABLE 1

Fatty Acid Composition (% total fatty acids) of the Dietary Fats Fed to Rats

Fatty acids	PHCO[a]	Canola oil
Sum saturated	8.4	7.7
8*t*-18:1	1.1	—
9*t*-18:1	7.3	—
10*t*-18:1	3.9	—
11*t*-18:1	2.8	—
12*t*-18:1	1.6	—
13*t*-18:1	1.0	—
14*t*-18:1	0.5	—
15*t*-18:1	0.4	—
16*t*-18:1	0.3	—
Sum *t*-18:1	18.9	—
6*c*-18:1	0.1	—
7*c*-18:1	0.5	—
8*c*-18:1	1.5	—
9*c*-18:1	46.3	55.1
10*c*-18:1	3.3	—
11*c*-18:1	4.3	3.6
12*c*-18:1	2.5	—
13*c*-18:1	0.3	—
14*c*-18:1	0.2	—
15*c*-18:1	0.3	—
16*c*-18:1	0.1	—
Sum *c*-18:1	59.4	58.7
9*c*,13*t*/8*t*,12*c*-18:2	1.9	—
9*c*,12*t*-18:2	1.1	0.1
8*t*,13*c*-18:2	0.5	—
9*t*,12*c*-18:2	1.0	0.1
10*t*,15*c*/9*t*,15*c*-18:2	1.1	—
tt-18:2	0.3	—
9*t*,12*t*-18:2	0.9	—
8*c*,13*c*-18:2	0.3	—
9*c*,13*c*-18:2	0.1	—
9*c*,14*c*-18:2	trace	—
9*c*,15*c*-18:2	0.9	—
12*c*,15*c*-18:2	trace	—
Sum unusual-18:2	7.6	0.2
18:2n-6	3.8	21.8
18:3n-3	trace	8.5
Sum EFA	3.8	30.3
t-18:3[b]	0.1	1.0
Others[c]	1.8	2.1
Sum *trans* fatty acids	26.6	1.2

[a]PHCO, partially hydrogenated canola oil; EFA, essential fatty acid.
[b]A mixture of mono-*trans* geometric isomers of 18:3n-3.
[c]Others include 16:1, 20:1, 22:1, 24:1 and 18:2 conjugated fatty acids.

Tissue PL fatty acids. The fatty acid composition of liver, heart, brain and serum PL is shown in Table 2. The gas chromatographic trace of the FAME profile of liver PL is shown in Figure 1. Feeding PHCO rats led to the incorporation of *c*- and *t*-18:1 and 18:2 isomeric fatty acids in liver, heart and serum. However, no dietary isomeric fatty acids were incorporated into brain. Generally, the fatty acid composition of brain PL was unaffected by that of the diet. Compared to the canola oil group, the liver, heart and serum PL of PHCO fed rats contained higher percentage of saturated fatty acids, 5*c*,8*c*,11*c*-20:3 and lower percentages of 18:2n-6, 22:5n-3 and 22:6n-3. Another noticeable difference was that although the content of 22:5n-6 in tissues of rats fed PHCO was quite large, it was almost absent in the canola oil group. In rats fed PHCO, as compared to the canola oil group, the content of 20:4n-6 was low in liver and serum PL. In contrast, the 20:4n-6 content of heart PL of PHCO was significantly higher than that in heart PL of canola oil group.

Comparison of the c- and t-18:1 isomer patterns of dietary PHCO (Table 1) with those in tissues of rats fed PHCO (Table 2) shows that isomers ranging from Δ11 to Δ16, especially the Δ12, were enriched in the tissue PL, relative to the lower Δ value isomers. This increase was accompanied by large decreases in the proportions of both 10c- and 10t-isomers. In the cis series, the proportion of 9c-18:1 was also lower in the tissue PL than in dietary PHCO. In the tissue PL of rats fed the canola oil, the 11c-18:1 isomer was the only significant component besides oleic acid.

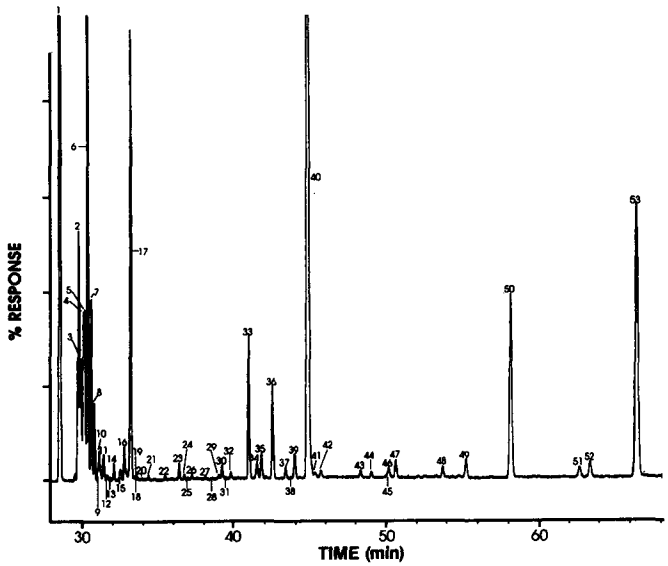

FIG. 1. The C_{18}, C_{20} and C_{22} regions of a gas chromatogram obtained on an SP-2560 capillary column (100 m × 0.25 mm) of the fatty acid methyl esters from liver phospholipids of rats fed partially hydrogenated canola oil. Peak identifications: (peak no., fatty acid) 1, 18:0; 2, 8c-9c-18:1; 3, 10t-18:1; 4, 11t-18:1; 5, 12t-18:1; 6, 13t-15t-18:1 + 6c-10c-18:1; 7, 11c-18:1; 8, 12c-18:1; 9, tt-18:2; 10, 13c-18:1; 11, 14c-18:1 + 16t-18:1; 12, tt-18:2 + 15c-18:1; 13, 9t,12t-18:2; 14, 9c,13t/8t,12c-18:2; 15, 9c,12t-18:2 + 8c,13c-18:2 + 9t,13c-18:2; 16, 9t,12c-18:2 + 10t,15c/9t,15c-18:2 + 9c,13c-18:2; 17, 18:2n-6; 18, 9c,14c-18:2; 19, 9c,15c-18:2; 20, unknown; 21, 12c,15c-18:2; 22, 20:0; 23, 11c-20:1; 24, 12c-20:1; 25, 18:2 conjugates; 26, 13c-20:1; 27, 18:2 conjugates; 28, 18:2 conjugates; 29, 11c,14t-20:2; 30, 8c,14c-20:2; 31, 11t,14c-20:2; 32, 20:2n-6; 33, 20:3n-9; 34, 5c,8c14c-20:3; 35, 5c,11c,14c-20:3; 36, 20:3n-6; 37, 5c,8c,11c,15t-20:4; 38, unknown; 39, 5c,8c,11c,14t-20:4; 40, 20:4n-6; 41, 22:1; 42, 22:1; 43, 22:2; 44, 22:2; 45, 20:5n-3; 46, unknown; 47, unknown; 48, 24:0; 49, 24:1; 50, 22:5n-6; 51, 22:5n-3; 52, t-22:6n-3; and 53, 22:6n-3.

TABLE 2

Fatty Acid Composition (% total fatty acids) in Liver, Heart, Serum and Brain Phospholipids of Rats Fed PHCO or Canola Oil[a]

	PHCO group				Canola oil group			
Fatty acids	Liver	Heart	Serum	Brain	Liver	Heart	Serum	Brain
Sum saturated	37.10 ± 3.22	25.16 ± 0.40	37.50 ± 1.20	44.41 ± 1.03	43.57 ± 1.01	37.92 ± 1.35	44.50 ± 0.44	44.34 ± 0.89
t-18:1								
8*t*-18:1	0.19 ± 0.02	0.23 ± 0.03	0.83 ± 0.10	—	—	—	—	—
9*t*-18:1	3.46 ± 0.41	1.89 ± 0.22	2.15 ± 0.31	—	—	—	—	—
10*t*-18:1	0.56 ± 0.07	0.86 ± 0.10	0.73 ± 0.21	—	—	—	—	—
11*t*-18:1	1.82 ± 0.21	1.23 ± 0.14	1.36 ± 0.18	—	—	—	—	—
12*t*-18:1	2.59 ± 0.30	1.06 ± 0.12	1.54 ± 0.27	—	—	—	—	—
13*t*-18:1	0.89 ± 0.10	0.36 ± 0.04	0.79 ± 0.07	—	—	—	—	—
14*t*-18:1	1.23 ± 0.14	0.42 ± 0.05	0.88 ± 0.12	—	—	—	—	—
15*t*-18:1	0.21 ± 0.02	0.20 ± 0.02	0.82 ± 0.11	—	—	—	—	—
16*t*-18:1	0.21 ± 0.02	0.12 ± 0.01	0.30 ± 0.07	—	—	—	—	—
Sum *t*-18:1	11.15 ± 1.30	6.37 ± 0.73	9.40 ± 1.43	—	—	—	—	—
c-18:1								
6*c*-18:1	—	—	0.20 ± 0.07	—	—	—	—	—
7*c*-18:1	0.04 ± 0.01	—	0.15 ± 0.04	—	—	—	—	—
8*c*-18:1	0.12 ± 0.01	0.42 ± 0.02	0.60 ± 0.12	—	—	—	—	—
9*c*-18:1	8.45 ± 0.81	10.09 ± 0.53	8.14 ± 0.94	20.25 ± 0.33	6.86 ± 0.07	8.77 ± 1.53	7.31 ± 0.29	19.30 ± 0.26
10*c*-18:1	0.09 ± 0.01	0.79 ± 0.04	0.82 ± 0.14	—	—	—	—	—
11*c*-18:1	2.38 ± 0.23	4.15 ± 0.22	2.21 ± 0.47	4.62 ± 0.36	3.17 ± 0.14	4.93 ± 0.17	2.71 ± 0.33	4.62 ± 0.25
12*c*-18:1	1.00 ± 0.10	1.55 ± 0.08	2.51 ± 0.39	—	—	—	—	—
13*c*-18:1	0.16 ± 0.01	0.17 ± 0.01	0.51 ± 0.08	—	—	—	—	—
14*c*-18:1	0.06 ± 0.01	0.09 ± 0.01	0.25 ± 0.07	—	—	—	—	—
15*c*-18:1	0.17 ± 0.02	0.17 ± 0.01	0.62 ± 0.12	—	—	—	—	—
16*c*-18:1	0.00 ± 0.00	0.05 ± 0.01	—	—	—	—	—	—
Sum *c*-18:1	12.43 ± 1.21	17.45 ± 0.93	16.01 ± 2.36	24.87 ± 0.65	10.03 ± 0.21	13.70 ± 1.57	10.02 ± 0.57	23.92 ± 0.28
Unusual-18:2								
9*c*,13*t*/8*t*,12*c*-18:2	0.26 ± 0.04	0.25 ± 0.08	0.45 ± 0.13	—	—	—	—	—
9*c*,12*t*-18:2	0.08 ± 0.05	0.18 ± 0.09	0.05 ± 0.08	—	0.01 ± 0.02	0.05 ± 0.05	0.04 ± 0.02	—
8*t*,13*c*-18:2	0.07 ± 0.03	0.07 ± 0.04	0.01 ± 0.02	—	—	—	—	—
9*t*,12*c*-18:2	0.45 ± 0.10	0.40 ± 0.11	0.81 ± 0.11	—	0.01 ± 0.01	0.08 ± 0.05	0.07 ± 0.02	—
10*t*,15*c*/9*t*,15*c*-18:2	0.01 ± 0.01	0.10 ± 0.05	0.08 ± 0.08	—	—	—	—	—
tt-18:2	0.04 ± 0.06	—	0.04 ± 0.02	—	—	—	—	—
9*t*,12*t*-18:2	0.01 ± 0.01	—	0.03 ± 0.01	—	—	—	—	—
8*c*,13*c*-18:2	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	—	—	—	—	—
9*c*,13*c*-18:2	0.01 ± 0.01	—	—	—	—	—	—	—
9*c*,14*c*-18:2	0.01 ± 0.01	—	0.01 ± 0.01	—	—	—	—	—
9*c*,15*c*-18:2	0.05 ± 0.02	0.18 ± 0.06	0.03 ± 0.01	—	—	—	—	—
12*c*,15*c*-18:2	0.01 ± 0.01	—	—	—	—	—	—	—
Sum unusual-18:2	1.02 ± 0.18	1.19 ± 0.27	1.52 ± 0.34	—	0.02 ± 0.03	0.13 ± 0.10	0.11 ± 0.04	—
Unusual C_{20} PUFA								
8*c*,14*c*-20:2	0.17 ± 0.04	0.10 ± 0.03	0.08 ± 0.04	—	—	—	—	—
11*c*,14*t*-20:2	0.02 ± 0.01	0.06 ± 0.01	0.10 ± 0.06	—	—	—	—	—
11*t*,14*c*-20:2	trace ± 0.01	0.01 ± 0.05	0.04 ± 0.03	—	—	—	—	—
5*c*,8*c*,14*c*-20:3	0.06 ± 0.05	0.13 ± 0.04	0.27 ± 0.10	—	—	—	—	—
5*c*,8*c*,11*c*,15*t*-20:4	0.18 ± 0.09	0.12 ± 0.05	0.56 ± 0.33	—	—	—	—	—
5*c*,8*c*,11*c*,14*t*-20:4	0.20 ± 0.05	0.20 ± 0.09	0.26 ± 0.24	—	—	—	—	—
Sum unusual C_{20} PUFA	0.63 ± 0.25	0.62 ± 0.22	1.31 ± 0.80	—	—	—	—	—
Normal PUFA								
Dead end pathway								
11*c*,14*c*-20:2	0.04 ± 0.05	0.06 ± 0.09	0.24 ± 0.12	—	0.16 ± 0.01	0.10 ± 0.02	0.12 ± 0.04	—
5*c*,11*c*,14*c*-20:3	0.26 ± 0.05	0.10 ± 0.02	0.06 ± 0.03	—	0.12 ± 0.04	0.20 ± 0.08	0.04 ± 0.01	—
5*c*,8*c*,11*c*-20:3	1.23 ± 0.21	1.05 ± 0.13	3.02 ± 0.55	—	0.08 ± 0.02	0.01 ± 0.01	trace	—

(continued on next page)

TABLE 2 (continued)

Fatty acids	PHCO group				Canola oil group			
	Liver	Heart	Serum	Brain	Liver	Heart	Serum	Brain
Main pathway								
18:2n-6	6.05 ± 0.73	9.44 ± 1.16	8.27 ± 0.63	0.60 ± 0.20	10.90 ± 0.17	20.70 ± 1.13	14.39 ± 0.17	0.83 ± 0.12
20:3n-6	0.93 ± 0.10	0.83 ± 0.13	1.55 ± 0.13	0.24 ± 0.07	1.29 ± 0.10	0.49 ± 0.07	1.57 ± 0.07	0.38 ± 0.09
20:4n-6	20.32 ± 2.40	26.76 ± 0.74	13.91 ± 1.60	9.63 ± 0.19	24.64 ± 1.20	17.09 ± 1.05	24.20 ± 0.41	10.06 ± 0.36
22:4n-6	0.16 ± 0.14	0.61 ± 0.06	0.04 ± 0.09	2.70 ± 0.25	trace	0.21 ± 0.06	trace	3.15 ± 0.31
22:5n-6	1.59 ± 0.46	2.28 ± 0.79	2.31 ± 0.43	0.88 ± 0.19	0.01 ± 0.01	0.09 ± 0.07	0.08 ± 0.03	1.06 ± 0.31
Sum n-6 PUFA	29.05 ± 3.83	39.92 ± 2.09	26.10 ± 1.30	14.30 ± 0.66	36.89 ± 0.07	38.71 ± 1.73	40.12 ± 0.60	15.48 ± 0.61
18:3n-3	0.19 ± 0.08	0.15 ± 0.10	0.01 ± 0.02	0.01 ± 0.01	0.20 ± 0.02	0.74 ± 0.03	trace	0.02 ± 0.01
20:5n-3	0.07 ± 0.02	0.29 ± 0.07	trace	trace	0.66 ± 0.34	0.11 ± 0.13	trace	0.04 ± 0.02
22:5n-3	0.12 ± 0.08	0.52 ± 0.05	trace	1.07 ± 0.24	0.66 ± 0.02	1.37 ± 0.33	0.03 ± 0.01	1.21 ± 0.28
22:6n-3	3.11 ± 0.59	6.89 ± 0.61	2.95 ± 0.45	11.45 ± 0.19	6.16 ± 0.13	6.53 ± 0.64	4.00 ± 0.34	11.17 ± 0.38
Sum n-3 PUFA	3.49 ± 0.77	7.85 ± 0.83	2.96 ± 0.47	12.53 ± 0.67	7.67 ± 0.44	8.75 ± 1.12	4.03 ± 0.34	12.44 ± 0.69
Others[b]	3.60 ± 0.63	0.23 ± 0.20	1.88 ± 0.17	3.89 ± 0.61	1.49 ± 0.37	0.62 ± 0.19	1.18 ± 0.22	3.84 ± 0.54

[a]Abbreviations as in Table 1. PUFA, polyunsaturated fatty acids. Trace, <0.01%. Values are mean ± standard deviation (n = 10).

Linoleic acid was the only 18:2 isomer in the PL in rats fed canola oil (Table 2). The 18:2 from liver PL of rats fed PHCO was largely linoleic, but it contained minor proportions (<0.8%) of several unusual isomers. The HPLC/$AgNO_3$–TLC and GC analyses suggested that all the unusual isomers originated from dietary PHCO. No new unusual isomers were detected. However, some pronounced quantitative differences in the individual isomers were observed. Among the unusual isomers, the relative proportions of 9c,12t-18:2, 8t,13c-18:2, 10t,15c/9t,15c-18:2 and 9c,15c-18:2 in particular were substantially lower in the tissue PL than in the dietary PHCO. On the other hand, 9t,12c was found in much higher proportions in the liver PL, and 9c,13t appeared to remain unchanged.

Feeding of PHCO diet to rats led to the formation of minor levels of six unusual C_{20} metabolites (peaks 29, 30, 31, 34, 37 and 39 in Fig. 1). They were identified as 11c,14t-20:2 (peak 29), 8c,14c-20:2 (peak 30), 11t,14c-20:2 (peak 31), 5c,8c,14c-20:3 (peak 34), 5c,8c,11c,15t-20:4 (peak 37) and 5c,8c,11c,14t-20:4 (peak 39). The mass spectra of the DMOX derivatives of those C_{20} PUFA fatty acids were consistent with the structures proposed for them. As an illustration, the mass spectrum of the DMOX derivative of 5c,8c,11c,15t-20:4, a hitherto unknown PUFA, is shown in Figure 2. Table 3 summarizes the results obtained for the other five unusual C_{20} PUFA. Only the diagnostic fragments, locating the double bond positions, are presented.

The mass spectrum of the DMOX derivative of 5c,8c,11c,15t-20:4 (Fig. 2) gave very intense ions at m/z 113 and 126, which are characteristic of the DMOX derivatives of fatty acids (18–20). The molecular ion appeared at m/z 357, which is in agreement with the molecular weight for the DMOX derivative of 20:4. The Δ5 double was localized by appearance of the prominent ion at m/z 153. This ion is a diagnostic ion in the spectra of DMOX derivatives with their first double bond at the Δ5 position (18–20). In DMOX derivatives of unsaturated fatty acids, a mass interval of 12 atomic mass units (amu) instead of the regular 14 amu, between two neighboring even-mass homologous fragments containing n-1 and n carbon atoms in the acid moiety indicates a double bond between n and n + 1 in the chain (18–20). Thus Δ8, Δ11 and Δ15 double bonds in 5c,8c,11c,15t-20:4 were clearly located by ions (m/z 180 vs. 192, m/z 220 vs. 232 and m/z 274 and 286) which differed by 12 amu. The prominent ion at m/z 260 confirms the presence of allylic bonds at the Δ11 and Δ15 positions. As shown in Table 3, the positions of the double bonds in the other unusual C_{20} were clearly localized by ions which differed by 12 amu and the Δ5 bond by a prominent ion at m/z 153.

The geometry of the double bonds in 5c,8c,11c,15t-20:4 could be deduced by comparison of the experimental and calculated ECL values. This approach has been success-

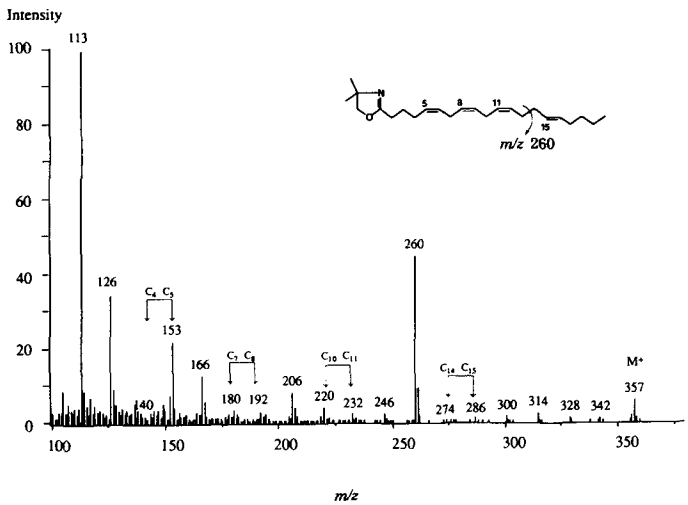

FIG. 2. Mass spectrum of the 2-alkenyl-4,4-dimethyloxazoline derivative of 5c,8c,11c,15t-20:4.

TABLE 3

Characteristic Ions in Mass Spectra of 2-Alkenyl-4,4-Dimethyloxazoline (DMOX) Derivatives of Five Unusual C_{20} PUFA

Fatty acid DMOX	M^+ m/z (intensity, %)	Diagnostic fragments m/z (intensity, %)
8*c*,14*c*-20:2	361 (5.4)	182 (11.9), 194 (6.8), 264 (2.3), 276 (2.6)
11*c*,14*t*-20:2	361 (6.7)	224 (9.4), 236 (5.6), 264 (2.1), 276 (1.7)
11*t*,14*c*-20:2	361 (5.9)	224 (10.1), 236 (6.3), 261 (2.4), 276 (2.0)
5*c*,8*c*,14*c*-20:3	359 (3.5)	153 (22.6), 180 (5.7), 192 (3.0), 262 (1.4), 274 (0.7)
5*c*,8*c*,11*c*,14*c*-20:4	357 (6.0)	153 (20.1), 220 (5.7), 222 (0.8), 232 (2.0) 234 (1.3), 260 (2.2), 272 (1.6)

fully applied to the identification of C_{20} *all cis*-polyunsaturated fatty acids (22), geometrical isomers of 20:5n-3 (23), as well as to some *cis* and *trans* C_{18} isomers (24). The observed ECL value for the FAME of the 5*c*,8*c*,11*c*,15*t*-20:4 isomer on the SP-2560 column was 22.46. It was possible to verify the ECL value of 22.46 for this isomer as the sum of the fractional chain length values of FAME of authentic 5*c*,8*c*,11*c*-20:3 and 15*t*-20:1, *ca.* 1.80 and 0.66, respectively, on the same GC column, plus the ECL base of 20.00, as there is no additional increment for interaction of the ethylenic bonds (25). Authentic 15*t*-20:1 (or 20:1n-5*t*) is not readily available. Therefore, its ECL value was estimated by extrapolating the retention time data of 13*t*-18:1 (or 18:1n-5*t*) using semi-log plots of retention time vs. carbon number, as described by Ackman (21). From all these data, the novel minor unusual 20:4 isomer was identified as 5*c*,8*c*,11*c*,15*t*-20:4. The structures of the other five unusual C_{20} PUFA were also identified using similar techniques.

The unusual C_{20} PUFA were mostly concentrated in the serum PL followed by liver and heart PL (Table 2). They were not detected in the brain PL nor in any of the tissues of rats fed canola oil. No unusual C_{22} metabolites were detected in any of the tissues examined.

DISCUSSION

The incorporation of dietary *c*- and *t*-18:1 isomers into tissue PL (except brain) indicates a preferential accumulation of high Δ value isomers, irrespective of their geometry. This phenomenon was also reported in rats fed hydrogenated soybean oil (26). Thus, the specificity for accumulation of 18:1 isomers appears to be more a matter of position of the double bond rather than of geometry. The observed large amounts both 12*c* and 12*t* isomers in tissue PL may indicate that the accumulation is more specific for the Δ12 position. None of the uncommon 18:1 isomers, except for 12*c*-18:1, were converted to C_{18} or C_{20} PUFA 18:2 (as discussed next). This suggests that all the uncommon 18:1 isomers, except for 12*c*-18:1, are not suitable substrates for chain elongation and/or desaturation. Furthermore, the specific accumulation of high Δ value isomers may indicate that high Δ value isomers are less readily subjected to β-oxidation than are low Δ value isomers. Within the series of *c*-18:1, the ratio of oleic acid to *cis*-vaccenic acid was lower in the tissues of rats fed PHCO than in rats fed canola oil. This may indicate that the incorporation of *c*-18:1 isomers into tissue PL is at the expense oleic acid rather than *cis*-vaccenic acid.

All the 18:2 isomers in the liver PL of rats fed PHCO originated from the diet, and no new isomers were detected. The isomer pattern was quite different from that reported by Holman *et al.* (8) for liver PL of rats fed partially hydrogenated soybean oil (PHSO). Almost all the unusual 18:2 isomers in that study were formed from the uncommon 18:1 isomers, either through Δ5 or Δ6 desaturation. The differences in the types and levels of the uncommon C_{18} PUFA in the two studies were most certainly due to the differences in the dietary levels of linoleic acid (5). The PHSO diet used by Holman *et al.* (8) was deficient in EFA and provided only about 0.16 energy% (en%) as linoleic acid. This allowed the uncommon isomers to act as substrates for chain desaturation. In the present study, this biochemical process was suppressed, probably because the linoleic acid content (1.46 en%) in the PHCO diet was adequate and above the EFA requirement of 0.6 en% for male rat (27).

The uncommon isomers of 18:2 also differ in the degree of their accumulation in tissue PL, with 9*t*,12*c*-18:2 and, to a lesser extent, 9*c*,13*t*/8*t*,12*c*-18:2 being more readily deposited than other dietary isomers. The selective accumulation of 9*t*,12*c* isomer over the 9*c*,12*t* isomer has also been reported by previous investigators (6,7,12). In studies with deuterated isotopes, the 9*t*,12*c* isomer became enriched in mouse liver 2–4 times more than the 9*c*,12*t* isomer (7). Of all the uncommon 18:2 isomers, only 9*t*,12*c*, 9*c*,13*t* and 9*c*,12*t* produced C_{20} PUFA metabolites (as discussed later). Thus, the lower accumulation of all the other isomers is indicative of their faster catabolic rates. We have also found a similar distribution of 18:2 isomers in adipose tissue of Canadian men (Chen, Z.Y., and Ratnayake, W.M.N., unpublished data) and in breast milk of lactating women (Chen, Z.Y., Pelletier, G., Hollywood, R., and Ratnayake, W.M.N., unpublished data).

Previous studies, using large amounts of synthetic 9*c*,12*t*-18:2 and linoleic acid, have shown that 9*c*,12*t*-18:2 is elongated to 5*c*,8*c*,11*c*,14*t*-20:4 similar to the conversion of linoleic acid to arachidonic acid (6,7,12). Furthermore, these reports have shown that *trans,trans* and the *trans,cis* geometric isomers of linoleic acid are not readily converted to isomers of arachidonic acid. The results of the present study confirm these metabolic pathways of the three geometrical isomers of linoleic acid. The 5*c*,8*c*,11*c*,14*t*-20:4 was the only geometrical isomer of arachidonic acid detected in all the tissue PL examined, except for brain PL. Beyers and Emken (7), using deuterated fatty acids, estimated that the rates of conversion of 9*c*,12*t*-18:2 and linoleic acid to 14*t*-20:4 and arachidonic acid were identical. In the present study, however, the 14*t*

geometrical isomer was a minor component in the tissue PL and a rapid desaturation and chain-elongation of 9c,12t-18:2 was not apparent. The ratio of 9c,12t-18:2 to linoleic acid in PHCO was 0.29, but the ratios of their products in the tissue PL were extremely low, i.e., 0.03, 0.02 and 0.03 for liver, heart and serum, respectively. This indicates that conversion of 9c,12t-18:2 to t-20:4 is lower than that of linoleic to arachidonic acid and that the reaction rates established using deuterated fatty acids (7) may not be applicable to diets containing an assortment of isomers, such as those present in PHVO. Although there was no large difference in the proportions of 9c,12t-18:2 and linoleic acid in the PHCO diet, they were not proportionately incorporated into the tissue PL. Linoleic acid was a major component, whereas 9c,12t-18:2 was only a minor component in the tissue PL. This may indicate that 9c,12t-18:2 was rapidly catabolized or was not absorbed as readily as was dietary linoleic acid. In any event, the higher proportion of linoleic acid in tissues provides a higher substrate level for the desaturase and elongase enzyme systems, which may partly explain the higher arachidonic level and lower 14t-20:4 level in tissues.

This is the first report that demonstrates the occurrence of 5c,8c,11c,15t-20:4 in animals fed PHVO. This unusual PUFA most likely derives from dietary 9c,13t-18:2 *via* an alternating sequence of desaturation, chain elongation and desaturation (Fig. 3) similar to the conversions of 9c,12t-18:2 to 5c,8c,11c,14t-20:4 (6,7,12) and 9c-18:1 to 5c,8c,11c-20:3 (28). Such a common biochemical pathway seems possible because the three precursors, 9c-18:1, 9c,12t-18:2 and 9c,13t-18:2, share some common structural features. The first double bond in these three fatty acids is at the Δ9 position and is in *cis* configuration. Thus, all three fatty acids seem to be ideal substrates for Δ6 desaturase (5). The *trans* double bond in both the 18:2 isomers is located between the Δ9c double bond and the methyl terminus, which imparts an extended structure toward the distal part of the fatty acid chain. Thus, the distal parts of the two 18:2 isomers share characteristics of a saturated hydrocarbon chain and thus are geometrically related to each other, as well as to 9c-18:1. We therefore suggest that 9c,12t-18:2 and 9c,13t-18:2 could biochemically mimic 9c-18:1, and that the three fatty acids could share a mutual metabolic pathway and produce structurally similar end products (Fig. 3). A similar explanation has been given by Wolff *et al.* (29) to rationalize the metabolical similarities between 9c,12c,15t-18:3 and 9c,12c-18:2. The intermediate metabolites shown in Figure 3 were not detected in the present study, suggesting transitory existence of the intermediate metabolites during the chain elongation and desaturation process of the three dietary fatty acids.

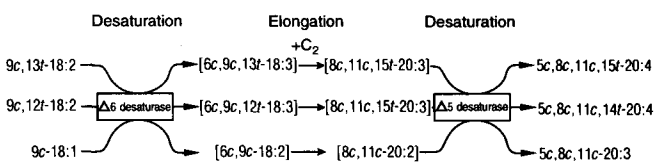

FIG. 3. Scheme showing the probable biochemical pathways for the formation of 5c,8c,11c,15t-20:4, 5c,8c,11c,14t-20:4 and 5c,8c,11c-20:3.

The two unusual mono-*trans* C_{20} diethylenic fatty acids, 11c,14t and 11t,14c, detected as trace components in tissues, were likely derived from the two mono-*trans* geometric isomers of linoleic acid, which were present in the dietary PHCO in almost equal quantities. In contrast to our findings, Beyers and Emken (7) found only 11c,14t-20:2, but not the other geometric isomer, in mice fed deuterated isotopes of the two mono-*trans* geometrical isomers. These authors suggested that 9t,12c-18:2, but not 9c,12t-18:2, is a good substrate for chain elongation. Our study, on the other hand, indicates that both are good substrates for chain elongation. Thus, it appears that the metabolism of the two mono-*trans* geometrical isomers of linoleic acid may be species dependent. Rats may have a greater capacity to metabolize isomeric fats from dietary sources than do mice. Other factors, such as the age of the animals and the duration of the experiments, could also have been responsible for the observed differences.

The two *all cis* NMI C_{20} PUFA, 8c,14c-20:2 and 5c,8c,14c-20:3, detected in the present study are structurally related to each other and, therefore, both PUFA may have originated from dietary 12c-18:1. Thus, 8c,14c-20:2 may arise by Δ6 desaturation of 12c-18:1, followed by 2-carbon chain elongation and subsequent Δ5 desaturation of 8c,14c-20:2 could give 5c,8c,14c-20:3. However, 6c,12c-18:2 was not present in the tissue PL, suggesting that the 2-carbon chain extension is the fastest step in the proposed metabolic pathway for 12c-18:1. The conversion of 8c,14c-20:2 to 5c,8c,14c-20:3 is probably similar to the conversion of 11c,14c-20:2 to 5c,8c,14c-20:3 (28). The latter two C_{20} PUFA are metabolites of the "dead end" metabolic pathway of linoleic acid (7). The levels of these four C_{20} PUFA are low in tissue PL, indicating that the previously mentioned pathways of 12c-18:1 and linoleic acid do not play a major biosynthetic role.

The amount of dietary linoleic acid has been implicated to exert a profound influence on the formation of unusual C_{20} PUFA in animals fed PHVO. Holman *et al.* (8) reported the formation of high levels of several C_{20} PUFA derived from isomeric fatty acids of oleic acid in rats fed an EFA-deficient PHSO diet. On the other hand, Zevenbergen *et al.* (30) did not report the occurrence of unusual PUFA in rats given PHSO containing 2 en% linoleic acid and various levels of *trans* fatty acids. The present study, however, shows the formation of unusual PUFA, even at adequate dietary levels of linoleic acid. Thus, it appears that linoleic acid at a dietary level of slightly greater than 1.46 en% is required to completely inhibit the formation of unusual C_{20} PUFA in rats. The presence of high levels of 22:5n-6 in tissue PL in the PHCO group indicated that the PHCO diet was deficient in the n-3 fatty acids, but not in n-6 fatty acids (31). The PHCO diet contained only trace levels of α-linolenic acid (<0.01% of total fatty acids), whereas the α-linolenic acid requirement in rats has been suggested to be 0.4% of the total dietary energy (33). This deficiency may have also exacerbated the formation of unusual PUFA, as all the dietary fatty acids compete with each other for the same desaturation and elongation enzymes. Future studies should focus on the optimal dietary

levels of linoleic and α-linoleic acids required to completely inhibit the formation of unusual PUFA. From the present study, it is apparent that of the various uncommon isomers of 18:1 and 18:2 in partially hydrogenated fats, 12*c*-18:1, 9*c*,12*t*-18:2, 9*t*,12*c*-18:2 and 9*c*,13*t*-18:2 are the preferred substrates for desaturation and chain elongation to C_{20} PUFA metabolites in the rat. However, their conversion to the C_{20} PUFA is not as efficient as that of oleic or linoleic acid.

It is well established that geometrical isomers of linoleic acid are incorporated, desaturated and elongated in the developing brain of mouse (7) and rat (11). Formation of *trans* long-chain n-3 PUFA has also been shown in the brain PL of developing rats (32). However, in the present study the dietary 18:1 and 18:2 isomers and their C_{20} PUFA metabolites were not detected in the brain PL. Furthermore, the PHCO diet had no overt influence on the fatty acid composition of the brain. In contrast to other studies, the present study was done on adult rats. Thus, it appears that the adult brain selectively excludes isomeric fatty acids, whereas the developing brain is less selective (11). In a separate study, we found significant amounts of *trans* and 18:2 isomeric fatty acids of PHVO origin in breast milk of lactating Canadian women (Chen, Z.Y., Pelletier, G., Hollywood, R., and Ratnayake, W.M.N., unpublished data). Future studies should investigate the possible incorporation of the isomeric fatty acids into the brain of infants and evaluate the physiological effects of such incorporation.

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Rat Liver Chromatin Phospholipids

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To shed light on the question whether the phospholipids present in chromatin are native or are due to contamination from nuclear membranes, we labeled the phospholipids of isolated nuclei and determined the amount of phospholipids (PL) and PL fatty acid composition in nuclei and chromatin. The hepatocyte nuclei were isolated and radioiodinated by the lactoperoxidase method under saturating and nonsaturating conditions, and the radioactivity associated with chromatin extracted from these nuclei was monitored. Whereas 97% the label was recovered in the nuclear membranes, only 0.08–0.6% was found in chromatin. The PL present in chromatin were relative to the amounts present in the entire nuclei and calculated as percentage of total, phosphatidylethanolamine (10%), phosphatidylserine (22%), phosphatidylinositol (19%) phosphatidylcholine (14%), and sphingomyelin (35%). In sphingomyelin of chromatin-associated PL an enrichment in polyunsaturated fatty acids was seen. The data indicated that the PL found in isolated chromatin do not seem to be due to contamination from the nuclear membrane.
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The presence of lipids inside the nuclei has been demonstrated in plant and animal cells using the acid hematein method (1). These results have been confirmed using autoradiography on semi-thin sections of plant cells incubated with [^{3}H]ethanolamine (2). Rees *et al.* (3) have shown that in liver nuclei, after complete destruction of the nuclear membrane, the fraction heavily labeled with [^{14}C]orotic acid contains phospholipids (PL) and probably corresponds to the etherochromatin attached to the nucleoli of whole nuclei. The presence of PL in chromatin was confirmed by Cocco *et al.* (4) after treatment with Triton X-100 and digestion of the nuclei with micrococconuclease. The results suggest that the PL are part of nuclear matrix and serve as anchorage for DNA during replication or are involved in the transport of mRNA within the nuclei (4). In blood lymphocytes, PL are linked to the nonhistonic fraction (5), and this complex has a stimulating effect on inactive chromatin extracted from thymus lymphocytes (6). A specific role has been attributed to sphingomyelin (SP), present in high concentrations in the nonhistonic fraction (5), which stimulates DNA polymerase activity *in vitro* (7) and inhibits RNA polymerase, which, in turn, is stimulated by phosphatidylserine (PS) (8). Changes in the concentration of SP have also been observed in tumor cells (9,10).

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Abbreviations: DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; GO, glucose oxidase; LPO, lactoperoxidase; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid(s); PMSF; phenylmethylsulfonylfluoride; PS, phosphatidyl-serine; SP, sphingomyelin; TLC, thin-layer chromatography.

These results have been criticized by Tata *et al.* (11) who showed that the presence of PL depends in part on cytoplasmic and in part on internal nuclear membrane contamination since their turnover is equal to that of the nuclei. Therefore, in spite of some evidence, the presence of PL in chromatin has often been considered a marker of incomplete purification (12). Nevertheless, the presence of PL in chromatin was corroborated using a method for separating chromatin from Triton X-100-treated hepatocyte nuclei, since chromatin had a PL and fatty acid composition different from that of the Triton X-100-treated whole nuclei (2). Turnover and synthesis of this fraction also differed from those of the nuclei and the microsomes (13). Autoradiography and electron microscopy have furthermore suggested the presence of PL inside the nuclei (14,15). Using gold-complexed phospholipase A_2 and RNAse, it has been shown that PL and RNA are present in noncondensed chromatin in hepatocyte nuclei (14) and in erythroleukemic cells (15). It has been demonstrated that during hepatic regeneration, chromatin PL are synthesized in parallel with DNA synthesis, whereas no modifications have been observed in the nuclear PL (16). There are also differences in the composition of nuclear and chromatin PL in proliferating rat liver cells after partial hepatectomy (17). The composition of PL furthermore changes in relation to changes in gene expression, as has been shown during hepatocyte maturation in newborns (18).

In view of the possible functional role of PL in chromatin (7–10), it is essential to demonstrate that they are not contaminants derived from nuclear membrane. This question was addressed in the present study in which the lactoperoxidase (LPO)-catalyzed radioiodination assay for lipids was employed. This method, initially used to study cell surface proteins (19), exclusively labelled the hydrophobic core of PL constituting the outer leaflet of liposomes or the surface of cultured cells (20,21). Therefore, labelling intact liver nuclei and following the radioactivity during chromatin preparation and PL extraction appears to be a suitable methodological approach to demonstrate whether the PL present in liver chromatin are derived from nuclear membrane contamination or not. Furthermore, comparison of the fatty acid patterns of the main PL classes found in chromatin with those of nuclear PL would provide further evidence of the specificity of chromatin-associated PL.

MATERIALS AND METHODS

Animals. Thirty-day-old Sprague-Dawley rats of either sex were used. They were kept on a natural light–dark cycle and had free access to pelleted food and water *ad libitum* prior to killing between 9 and 10 a.m.

Nuclei preparation and chromatin isolation (Procedure I). Rats were anesthetized, and livers were perfused

via the portal vein with 0.25 M sucrose containing 3 mM $CaCl_2$ and 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma, St. Louis, MO) adjusted to pH 7.4 with concentrated Tris. The livers were then quickly removed and washed in the same solution. Hepatic nuclei were isolated according to the procedure of Bresnick *et al.* (22) which yields 95–98% of isolated hepatocyte nuclei. After washing in the solution described by Barnes *et al.* (23), the nuclei were gently suspended in 10 mM Tris containing 0.25 M sucrose, 1 mM PMSF and 0.3% (vol/vol) Triton X-100 (Sigma) adjusted to pH 7.4 with 0.1 N NaOH, and were centrifuged for 5 min at 5,000 × *g* at 4°C. Swelling of the nuclei and chromatin extraction were done according to Shaw and Huang (24). The nuclei were then washed four times with hypotonic solution containing 0.075 M NaCl, 0.024 M ethylenediaminetetraacetic acid (EDTA), and 1 mM PMSF adjusted to pH 8 with 0.1 N NaOH, and again washed with Tris solution of decreasing concentrations (from 50 to 0.4 mM) containing 1 mM PMSF, pH 8. The entire procedure was carried out at 0–4°C. Finally, the material was suspended in 1 mM PMSF (100 mL for 20 g of liver), brought to pH 8 with NH_4OH, and stirred overnight at 4°C. The solution was then centrifuged at 90,000 × *g* for 30 min at 4°C. The chromatin-containing sediment was resuspended in 10 mM Tris, pH 8. During purification, the preparations were monitored at each step by light microscopy following methylene blue staining to check the swelling of nuclei and the chromatin separation; and also by measuring biochemical markers as previously described (2).

Preparation of nuclei and isolation of nuclear membranes (Procedure II). Rats were killed by cervical dislocation and livers were rapidly removed, chopped, and then nuclei isolated according to the procedure of Widnell and Tata (25). Briefly, tissue was homogenized in 0.32 M sucrose containing 3 mM $MgCl_2$, pH 7.4, and then centrifuged at 700 × *g* for 10 min at 4°C. The pellet was resuspended in the same medium, and centrifugation was repeated. The final pellet was resuspended in 2.4 M sucrose containing 1 mM $MgCl_2$, pH 7.4, and the suspension was centrifuged at 50,000 × *g* for 60 min at 4°C. The nuclei were then washed with 0.25 M sucrose containing 1 mM $MgCl_2$, pH 7.4, and the suspension was centrifuged at 700 × *g* for 5 min at 4°C. This procedure, although suitable for the preparation of membranes is not suitable for the isolation of chromatin. The nuclei isolated by this method are being referred to as liver nuclei.

Nuclear membranes were isolated according to Kay and Johnston (26). The isolated nuclei were lysed in 8 mM Tris HCl, pH 8.5, containing 0.1 mM $MgCl_2$, 4 mM 2-mercaptoethanol (Merck, Darmstadt, Germany), 8% sucrose, 1 μg/mL deoxyribonuclease I (DNase I) (Sigma). After 15 min of incubation at 22°C, the reaction was stopped by diluting the lysate with cold water (1:1, vol/vol). The lysed nuclei were centrifuged at 38,000 × *g* for 15 min at 4°C, and the sediment was resuspended in 10 mM Tris HCl, pH 7.4, containing 0.1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% sucrose and 1 μg/mL DNase I. Digestion was carried out for 20 min and stopped as described. The final nuclear envelope pellet was obtained after centrifugation at 38,000 × *g* for 15 min at 4°C.

LPO-catalyzed radioiodination of nuclei and chromatin. The LPO reaction (27,28) was used to radiolabel lipid moieties. For this purpose, hepatocyte nuclei (isolated by Procedure I) were suspended in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS), pH 7.2, and liver nuclei (obtained by Procedure II) were suspended in 0.25 M sucrose containing 1 mM $MgCl_2$. In both experiments the nuclear suspension contained 5 mg protein, 1.1–1.2 mg DNA and 0.6–0.7 mg RNA. Under nonsaturating conditions, the reagents were added to 1 mL of nuclei suspension which included 400 μCi $Na^{125}I$ (212 pmol) free of carrier (15 mCi/μg ^{125}I; Amersham, Buckinghamshire, United Kingdom) in 400 μL PBS, 50 μg LPO [EC 1.11.1.7.] (Sigma) in 50 μL PBS, and 50 mU of glucose oxidase (GO) [EC 1.1.3.4.] (Sigma) in 50 μL PBS. The reaction was initiated by adding 45 μg D(+)glucose (Merck) in 50 μL PBS and then allowed to proceed for 10 min at room temperature. Under saturating conditions, the carrier-free radioactive iodide was mixed with 240 nmol KI Analar grade (Prolabo, Paris, France) and 100 μg LPO (in 50 μL PBS) and 500 mU GO (in 50 μL) were added. The reaction was initiated by adding 450 μg D(+)glucose and continued for 30 min at room temperature.

After radioiodination the lipids of 100 μL of the nuclear suspension were extracted. The remaining labeled nuclei were diluted with unlabeled nuclei for other assays. All experiments were repeated three times for each of the isolation procedures.

The method was also used for chromatin, except that the LPO and GO concentrations were increased five- and twofold, respectively, in respect to the amount of protein used.

Lipid extraction. Lipids were extracted from nuclei (8 mg/mL) and chromatin (2.5 mg/mL) with 20 vol of chloroform/methanol (2:1, vol/vol). The organic phase was washed with 0.2 vol 0.74% KCl, and after phase partition the organic phase was washed with 0.4 vol of chloroform/methanol/water (3:48:47, by vol) according to Folch *et al.* (29). When radioiodinated PL were extracted, the washing phases contained 1 mM KI.

Radioiodinated lipid fractionation. The organic phase was evaporated under a nitrogen stream and fractionated on silica H 60 (Merck) columns (35 mm high, 3 mm diameter; bed volume, 0.3 mL). The silica was washed once with chloroform/methanol (1:1, vol/vol), twice with methanol, and then activated at 110°C. From the silica equilibrated in chloroform, the neutral lipids, the neutral glycolipids, and the PL were eluted successively with 4 volumes of chloroform, acetone, and methanol, respectively. The radioactivity of each of the fractions was determined.

PL identification and fatty acid analysis. PL were separated on thin-layer chromatography (TLC) plates (Whatmann LK5, Maidstone, United Kingdom) according to Leray *et al.* (30). The plates were pretreated with 2.3% boric acid in ethanol and activated for 15 min at 110°C. Chloroform/ethanol/water/triethylamine (30:35:7:35, by vol) was used as solvent. The plates were air-dried and sprayed with primulina (5 mg/100 mL acetone/water, 4:1, vol/vol), and the PL were identified under ultraviolet light relative to standards. The PL were then subjected to

boron trifluoride (Sigma)-catalyzed methanolysis without prior elution according to Morrison and Smith (31). Fatty acid methyl esters dissolved in *n*-hexane were analyzed using a Perkin-Elmer Sigma I gas chromatograph (Palo Alto, CA) equipped with a 50-m bonded fused silica open tubular column (0.32 mm diameter × 50 m, superox; Altech Associates Inc., Deerfield, IL) with helium gas as carrier (23 psi) and at a column temperature of 200°C. The split/splitless injector and the flame-ionization detector were maintained at 230°C. The amounts of PL were estimated using methylheptadecanoate as internal standard and are expressed as nmol PL/mg DNA. Abundances of individual fatty acids are given in mole percent relative to total fatty acid methyl esters.

Other assays. Protein, DNA and RNA contents were determined according to Lowry *et al.* (32), Burton (33) and Schneider (34), respectively. Since the RNA values were not corrected for the presence of DNA, the RNA values are overestimated about 1.4-fold when the DNA concentration is five times that of RNA, as it is in chromatin (34).

RESULTS

Criteria of purity for isolated chromatin and nuclear membranes. Rat liver tissue contains hepatocytes as well as other cell types, including Kupffer and endothelial cells and connective tissue which represent about 40% of the total (35). When using Procedure I, 95–98% of the nuclei recovered were from hepatocytes, whereas Procedure II yielded 60% hepatocyte and 40% nonhepatocyte nuclei. Therefore nuclei from Procedure I are being referred to as hepatocyte nuclei, those from Procedure II as liver nuclei, although no significant differences in DNA and RNA contents, expressed in μg/mg protein were seen between the preparations (Table 1). The isolated nuclear membrane was found to contain approximately the same amount of DNA and RNA/mg protein (Table 1), which is in agreement with data reported by Kay and Johnston (26).

Chromatin isolated from hepatocyte nuclei contained a significantly greater amount of DNA/mg protein than did hepatocyte and liver nuclei preparations (Table 1). This difference is also evident when one compares the percentages of total DNA (Table 1). Our present results are similar to those obtained in a previous study (2) on hepatocyte chromatin of rats of different age (28–32% DNA, 5–7% RNA, 60–65% protein and 0.2% PL). In the hepatocyte nuclei, the RNA/DNA ratio was 0.32 and decreased by 35% to 0.21 upon chromatin isolation. This decrease is similar to that reported by Tata *et al.* (11) and gives additional credence to the purity of the preparations.

LPO-catalyzed radioiodination. Table 2 shows that a 1100-fold dilution of radioactive iodide ($Na^{125}I$) with KI reduced the amount of label associated with nuclear PL (8-fold and 6-fold in nuclei isolated by Procedures I and II, respectively). Thus, under saturating conditions, all PL accessible to the radioiodination system appeared labelled. Under nonsaturating conditions, at least 10% of the sites accessible to LPO were labelled. The high specific radioactivity allowed for a more accurate identification of the label in the course of the chromatin and nuclear preparations. It is noteworthy that the labelling of nuclei isolated according to Procedure II was 96% lower than for nuclei isolated according to Procedure I. The low labeling yield could be explained by the fact that the nuclei were labeled in unbuffered medium containing Mg^{2+} (4 mM), which is known to inhibit LPO (36).

Table 2 also shows that the label associated with nuclear PL was almost entirely recovered in the nuclear membranes, while only minute quantities of radioactivity were found in the PL extracted from chromatin (0.08–0.6%). Extraction of labeled PL from either nuclei or nuclear membranes, followed by TLC separation and autoradiography, indicated that all the major PL classes were radioiodinated. Since the amounts of labeling seen in chromatin are very similar, irrespective of whether saturating or nonsaturating radioiodination conditions were used with intact nuclei, the minute quantities of labeling are probably due to adsorbed free radioactive iodine which migrates with the solvent front on the chromatogram. When chromatin was isolated from unlabeled nuclei and

TABLE 1

Comparison of DNA and RNA Contents of Hepatocyte Nuclei, Chromatin, Liver Nuclei and Nuclear Membranes[a]

	Hepatocyte nuclei	Chromatin	Liver nuclei	Nuclear membranes
DNA	210.7 ± 22.8[b] (16.4 ± 1.5)	351.7 ± 67.5[b] (24.4 ± 3.5)	230.5 ± 70.0[c] (17.3 ± 3.9)	55.2 ± 30.4[c] (7.0 ± 1.4)
RNA	69.0 ± 30.0 (5.3 ± 2.1)	77.6 ± 21.7 (5.4 ± 1.3)	93.8 ± 44.8 (10.5 ± 3.1)	54.0 ± 37.2 (7.0 ± 1.0)

[a]Values are expressed as μg/mg protein and indicate means ± SD of ten experiments for hepatocyte nuclei, 15 for chromatin, 3 for liver nuclei and 3 for nuclear membranes. Values given in parentheses are percentage of total nuclear content.
[b]$P < 0.05$ for difference between hepatocyte nuclei and chromatin.
[c]$P < 0.05$ for difference between liver nuclei and nuclear membranes.

TABLE 2

Distribution of ^{125}I-Label Associated with Phospholipids Extracted from Nuclei, Nuclear Membranes and Chromatin[a]

	Hepatocyte nuclei	Chromatin	Liver nuclei	Nuclear membranes
Nonsaturating conditions[b]	7084×10^3	6×10^3 (0.08%)	208×10^3	202×10^3 (97%)
Saturating conditions[c]	873×10^3	5×10^3 (0.6%)	38×10^3	36×10^3 (95%)

[a]Values for a typical experiment are given in cpm/mg protein. The recoveries of label associated with phospholipids that were extracted from chromatin and from nuclear membranes are given in parentheses. The experiment was repeated four times. Nuclei were separated according to the method of Bresnick *et al.* (22) for hepatocytes and of Kay and Johnston (26) for liver nuclei. Nuclei were radioiodinated using lactoperoxidase under saturating and nonsaturating conditions. Chromatin was extracted according to the method of Shaw and Huang (24).
[b]1.6×10^{-3} mol Na ^{125}I/mol phospholipid.
[c]Footnote c as footnote b + 1.8×10^{-3} mol K ^{125}I/mol phospholipid.

TABLE 3

Comparison of the Amounts of Total Phospholipid (PL) and of Individual PL Classes in Hepatocyte Nuclei and Chromatin[a]

	PL	PE	PS	PI	PC	SP
Nuclei	123.9 ± 10.1	25.6 ± 2.3	3.2 ± 0.5	14.5 ± 2.4	68.5 ± 13.2	6.5 ± 0.5
Chromatin	12.5 ± 3.6	2.5 ± 0.8	0.7 ± 0.3	2.8 ± 0.7	9.5 ± 0.5	2.4 ± 1.0
	(10)	(9.8)	(22)	(19)	(14)	(35)

[a]Values are given in nmol/mg DNA. Values in parentheses give the percentage of individual chromatin PL classes relative to total nuclear PL. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SP, sphingomyelin.

then radioiodinated, intense labeling was seen which is contrary to the results obtained with chromatin extracted from radioiodinated nuclei.

As the data show that chromatin PL extracted from labelled nuclei are unlabelled, and PL are labelled only when the radioiodination is done directly on chromatin, the chromatin PL cannot be due to lipid contamination of the nuclear membrane PL.

Principal PL distribution and fatty acid pattern of nuclei and chromatin. The total PL content of hepatocyte nuclei was 124 nmol/mg DNA as illustrated in Table 3. PL in the chromatin amounted to 12.5 nmol/mg DNA (Table 3), which was about 10% of the amount present in the nuclei and is in agreement with previous data (2).

If the nmoles of individual chromatin PL classes are divided by the respective nuclear total PL (considered as equal to 100), phosphatidylethanolamine (PE) amounts to 9.8%, PS to 22%, phosphatidylinositol (PI) to 19%, phosphatidylcholine (PC) to 14% and SP to 35% (Table 3). This clearly shows that compared to nuclei PL, chromatin PL are particularly rich in SP, but are deficient in PE.

Fatty acid analyses showed a high unsaturated/saturated fatty acid ratio for chromatin PE largely due to a low 16:0 level (Table 4). PS showed a decrease in 18:1, but an increase in 20:4 and 22:6 relative to PS of the nuclei. Large differences were observed for chromatin SP, which was rich in polyunsaturated fatty acids.

The data taken together are consistent with the idea that chromatin-associated PL have specific fatty acid profiles.

DISCUSSION

LPO-catalyzed radioiodination of liver nuclei and localization of the label showed a 97% recovery in nuclear membrane PL and of 0.08–0.6% in chromatin PL. A compari-

TABLE 4

Fatty Acid Distribution in Phospholipids Extracted from Rat Liver Nuclei and Chromatin[a]

Fatty acid	Source	PE	PS	PI	PC	SP
16:0	Nuclei	35.1 ± 3.4	19.6 ± 2.9	17.7 ± 6.9	34.8 ± 5.6	27.2 ± 6.6
	Chromatin	25.3 ± 3.9	14.6 ± 3.6	15.3 ± 3.9	34.8 ± 3.6	36.7 ± 2.7
18:0	Nuclei	15.3 ± 2.0	28.3 ± 5.6	25.8 ± 4.5	12.6 ± 4.5	10.8 ± 1.5
	Chromatin	18.0 ± 5.2	31.5 ± 5.7	29.1 ± 2.9	12.6 ± 1.2	13.3 ± 1.9
18:1n-9	Nuclei	5.3 ± 0.6	19.3 ± 3.8	6.3 ± 0.5	7.6 ± 0.5	29.0 ± 10.0
	Chromatin	8.2 ± 3.8	7.5 ± 2.7	8.1 ± 1.1	7.8 ± 1.2	20.6 ± 5.0
18:2n-6	Nuclei	8.2 ± 1.4	3.7 ± 0.9	4.3 ± 0.8	14.6 ± 1.9	6.1 ± 2.2
	Chromatin	9.2 ± 1.1	5.5 ± 0.6	4.5 ± 0.5	14.8 ± 0.8	5.1 ± 0.3
18:3n-6	Nuclei	0.2 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.2	0.9 ± 0.8
	Chromatin	1.0 ± 0.2	1.3 ± 0.3	0.6 ± 0.1	0.5 ± 0.1	1.9 ± 0.2
20:4n-6	Nuclei	22.4 ± 2.0	10.7 ± 0.1	30.4 ± 2.1	13.6 ± 1.6	0.2 ± 0.2
	Chromatin	16.7 ± 2.9	15.2 ± 0.2	26.4 ± 2.9	12.6 ± 1.8	2.6 ± 0.6
22:6n-3	Nuclei	5.3 ± 1.2	2.8 ± 1.4	1.6 ± 0.6	2.4 ± 0.8	0.9 ± 1.5
	Chromatin	2.7 ± 0.5	7.9 ± 1.0	1.0 ± 0.1	2.4 ± 1.7	2.2 ± 1.9
Unsaturated/ saturated ratio	Nuclei	1.09 ± 0.26	1.12 ± 0.20	1.12 ± 0.20	1.02 ± 0.04	0.85 ± 0.22
	Chromatin	1.19 ± 0.04	1.00 ± 0.10	1.45 ± 0.50	1.07 ± 0.13	0.69 ± 0.15

[a]Results are expressed as mole percentage of total fatty acid methyl esters; only major components are listed. For abbreviations see Table 3.

son of data obtained under nonsaturating and saturating labeling conditions showed that chromatin labeling was due to free iodide rather than to contamination by nuclear membrane. Since chromatin PL amounted to 10% of the total nuclear PL and contamination was less than 1%, this would support the conclusion that the PL extracted from chromatin are genuine chromatin PL and are not due to PL contamination. These data are in agreement with electron microscopic evidence for the presence of PL inside the nuclei based on gold labelling (14,15). The lipids appear largely localized in the active chromatin (6), and their distribution seems similar to that of RNA (14,15). The PL appear more concentrated near the nucleolus and, according to Cocco *et al.* (4), are likely to be anchored close to the nuclear matrix.

Contamination can also be excluded because of the decreases seen in RNA and PL with respect to the nuclei. In fact, the RNA/DNA ratio seen in chromatin was 35% lower than that in the nuclei, a difference similar to that reported by Tata *et al.* (11). Previous studies on PL composition, synthesis and turnover (2,16,13) had indicated that the PL found in chromatin are not contamination artifacts, but constituents of chromatin. Moreover, nuclear membrane or cytoplasmic contamination does not fit the time course for ^{32}P incorporation into chromatin (13). Our present data provide direct and independent evidence in support of our previous findings based on different methodologies.

Analyses of the PL composition of the nuclei show that chromatin is particularly rich in SP which confirms the earlier observation by Chayen and Gahan (37). Fatty acid analyses confirm that chromatin PL differ from whole nuclei PL. In particular, SP is rich in polyunsaturated fatty acids and all PL being components of chromatin have specific profiles.

In regard to the role of chromatin PL, several hypotheses about their potential function in relation to DNA structure and translational activity have been proposed (7). However, at present there is no clear evidence for their specific role *in vivo*. During rat liver regeneration, *de novo* synthesis of chromatin-associated PL was correlated with DNA replication, whereas this was not the case for nuclear PL (16). Changes in PL composition were observed, especially during the first days of life (18) at the onset of hepatocyte maturation (38).

In regard to PL synthesis, the kinetics of ^{32}P incorporation in chromatin were shown to be similar to those found in microsomes, except that its peak was delayed by three hours (13), suggesting that PL are synthesized in microsomes from where they are transferred to chromatin (13). Experiments are presently under way to explore these questions.

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METHOD

Preparation of Defined Molecular Species of Lactosylceramide by Chemical Deacylation and Reacylation with *N*-Succinimidyl Fatty Acid Esters

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A procedure for the preparation of specific molecular species of D-*erythro*-lactosylceramide involving deacylation and reacylation of lactosylceramide prepared from bovine brain gangliosides is described. Lactosylceramide was *N*-deacylated by alkaline hydrolysis and the resulting four lysolactosylceramides, which contained d18:1, d20:1, d18:0 and d20:0 long-chain bases, were simultaneously re-*N*-acylated with the *N*-succinimidyl ester of either 16:0, 18:0, 20:0, 22:0, 24:0, 20:1, 22:1 or 24:1 fatty acid. The resulting lactosylceramide contained four molecular species of lactosylceramides, i.e., d18:1, d20:1, d18:0 and d20:0 long-chain bases coupled with the fatty acid that was introduced. Lactosylceramides prepared in this manner were separated into four individual molecular species by high-performance liquid chromatography (HPLC). Each of the purified molecular species of lactosylceramide was quantitated by HPLC after derivatization with benzoylchloride and was characterized by mass spectrometry. The yields of reacylated lactosylceramide were 38–58% relative to the starting lactosylceramide; the purity of each of the molecular species of lactosylceramide was greater than 95%.

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The ceramide molecular species composition of various glycosphingolipids has been shown to affect the biological activity of glycosphingolipids (3–5). In the case of the intrinsic Golgi membrane enzyme CMP-*N*-acetylneuraminate:lactosylceramide α2,3-sialyltransferase (LacCerα2,3-ST) [EC 2.4.99.9], which catalyzes the synthesis of (*N*-acetylneuraminosyl)-galactosylglucosylceramide (GM3) from lactosylceramide (LacCer), it has been shown that the enzyme displays a distinct molecular species specificity for the lipophylic substrate LacCer (6,7) and that the molecular species specificity of the enzyme is highly dependent on the lipid composition of the Golgi membrane (8). To determine the basis for this effect, it became necessary to obtain a wide range of pure molecular species of LacCer containing specific combinations of long-chain base and fatty acid. It is difficult to isolate individual pure molecular species of LacCer from natural sources because the LacCer content is generally very low and the molecular species composition of most LacCer is highly complex. LacCer can be prepared from other glycosphingolipids by stepwise enzymatic hydrolysis or by mild acid hydrolysis, but it is still not possible to isolate more than a few individual molecular species of LacCer from natural sources. It therefore became necessary to chemically synthesize the specific LacCer molecular species required to shed light on the molecular species specificity of LacCerα2,3-ST. The present paper describes the synthesis of pure molecular species of LacCer by *N*-deacylation of LacCer (derived from bovine brain gangliosides) followed by re-*N*-acylation of the resulting lyso-LacCer with the *N*-succinimidyl ester of one of several fatty acids.

EXPERIMENTAL PROCEDURES

Materials. Octadecanoic, eicosanoic, docosanoic, tetracosanoic, *cis*-11-eicosenoic, *cis*-13-docosenoic and *cis*-15-tetracosenoic acids were obtained from Nu-Chek-Prep (Elysian, MN); *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, palmitic acid *N*-hydroxysuccinimide ester, psychosine (1-β-D-galactosylsphingosine) and triethylamine were from Sigma Chemicals (St. Louis, MO); silica gel 60 (particle size 0.063–0.100 mm) and precoated high-performance thin-layer chromatography (HPTLC) plates coated with silica gel 60 were from EM Separations (Gibbstown, NJ); DEAE-Sephadex A25 was from Pharmacia Fine Chemicals (Piscataway, NJ); analytical and HPLC-grade solvents, and Nylon membrane filter (pore size 0.45 μm) were from Fisher Scientific (Medford, MA); bovine brain was from Pel-Freez Biologicals (Rogers, AR). Globotetraosylceramide ($GbOse_4Cer$) was prepared from human red blood cells (9).

Synthesis of N-succinimidyl ester of fatty acid. The *N*-succinimidyl ester of each of the fatty acids used (i.e., 18:0, 20:0, 22:0, 24:0, 20:1, 22:1 and 24:1) was synthesized by reacting the fatty acid with *N*-hydroxysuccinimide in

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Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LacCerα2,3-ST, CMP-*N*-acetylneuraminate:lactosylceramide α2,3-sialyltransferase; NeuAc, *N*-acetylneuraminic acid.

The glycosphingolipid nomenclature is as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1). GalCer, galactosylceramide, Gal(β1-1)Cer; GlcCer, glucosylceramide, Glc(β1-1)Cer; LacCer, lactosylceramide, Gal(β1-4)GlcCer; $GbOse_3Cer$, globotriaosylceramide, Gal(α1-4)Gal(β1-4)GlcCer; $GbOse_4Cer$, globotetraosylceramide, GalNAc(β1-3)Gal(α1-4)-Gal(β1-4)GlcCer; $GgOse_3Cer$, gangliotriaosylceramide, GalNAc-(β1-4)Gal(β1-4)GlcCer; $GgOse_4Cer$, gangliotetraosylceramide, Gal(β1-3)GalNAc(β1-4)Gal(β1-4)GlcCer; GM3, (NeuAcα2-3)-Galβ1-4GlcCer; GM1, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4-GlcCer.

The molecular species abbreviations suggested by Breimer *et al.* (2) are used. For example, in the notation d18:1-18:0, the d18:1 represents the long-chain base sphingosine (D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene) and 18:0 represents the fatty acid (octadecanoic acid).

the presence of *N,N'*-dicyclohexylcarbodiimide according to the method of Lapidot *et al.* (10). The purity of each of the recrystallized *N*-succinimidyl fatty acid esters was determined by HPTLC (10). Only a single spot corresponding to the *N*-succinimidyl fatty acid ester was observed in HPTLC.

Preparation of LacCer. LacCer was prepared from both gangliosides and $GbOse_4Cer$. Gangliosides were isolated from bovine brain as described by Ledeen and Yu (11) and Kadowaki *et al.* (12).

Approximately 100 mg of gangliosides are incubated with 6 mL of 0.05 N sulfuric acid in water at 80°C for 2 h, and the resulting hydrolysate is neutralized with sodium hydroxide. Chloroform and methanol are then added to bring the final solvent proportion to Folch partitioning conditions (13). After vigorous mixing, the two phases are separated by centrifugation. The upper phase contains gangliosides (predominantly GM1). The lower phase contains two major glycosphingolipids, i.e., gangliotriaosylceramide ($GgOse_3Cer$) and gangliotetraosylceramide ($GgOse_4Cer$), and two minor ones, i.e., glucosylceramide (GlcCer) and LacCer. The lower phase is evaporated to dryness, redissolved in chloroform, and applied to a silica gel 60 (acid-washed) column (3 × 25 cm) to isolate LacCer. The column is eluted successively with 1 L each of chloroform, chloroform/methanol (96:4, vol/vol), (94:6), (92:8), (90:10), (86:14) and finally methanol. The glycosphingolipid composition of each fraction is monitored by applying an aliquot to an HPTLC plate which is developed in chloroform/methanol/water (60:35:8, by vol) and on which fractions are visualized with orcinol spray (14). The fractions containing LacCer as the major component are combined, evaporated to dryness, dissolved in chloroform, and then re-applied to a silica gel 60 column (1 × 25 cm). The column is eluted successively with 100 mL of mixtures of chloroform and methanol (from 6 to 14% methanol in 1% increments). The glycosphingolipid composition of these fractions is also monitored by HPTLC as described above. The fractions containing only LacCer are combined and used for the deacylation procedure. Fractions from the silica gel 60 column that contain $GgOse_3Cer$ and $GgOse_4Cer$ are combined and subjected to further acid hydrolysis. Gangliosides in the upper phase are also subjected to further acid hydrolysis. LacCer obtained from these fractions is purified as described above. After several cycles of acid hydrolysis, approximately 25 mg of LacCer is obtained. LacCer was also prepared from human red blood cell $GbOse_4Cer$ by enzymatic cleavage of the sugars as previously described (15).

Preparation of lysoLacCer. LysoLacCer was prepared by a modified procedure developed for the preparation of lysocerebrosides by Radin (16). For this purpose, LacCer (10 μmol) is dissolved in 3.6 mL of *n*-butanol in a culture tube (20 × 150 mm) with a Teflon-lined cap, and 0.4 mL of 12.5 M potassium hydroxide in water is added. The reaction mixture is sealed under nitrogen and heated at 125°C for 4 h. After cooling, 10.4 mL of chloroform, 1.2 mL of methanol and 2.4 mL of water are added to the reaction tube. The tube is shaken vigorously and then centrifuged at 1,000 × *g* to separate the two phases. The lower phase (containing lysoLacCer) is washed twice with 2.5 mL of saline/methanol (1:1, vol/vol) and dried under a stream of nitrogen.

Re-N-acylation of lysoLacCer and purification of the product LacCer. LysoLacCer is re-*N*-acylated with *N*-succinimidyl fatty acid ester in the presence of triethylamine by a modification of the procedure for the reacylation of lysoGM1 ganglioside as reported by Neuenhofer *et al.* (17). *N*-Succinimidyl fatty acid ester (50–200 μmol) and lysoLacCer (derived from 10 μmol of the original LacCer) are dissolved in 10 mL of chloroform/methanol/water (60:40:9, by vol) in a culture tube (16 × 125 mm) with a Teflon-lined cap, and 100 μL of triethylamine is added. The tube is sealed under nitrogen, and the reaction is allowed to proceed overnight at room temperature. To the reaction mixture, 2 mL of chloroform and 2 mL of water are added, mixed vigorously, and then centrifuged at 1,000 × *g* for 10 min. The lower phase containing reacylated lysoLacCer is washed twice with 2 mL of saline/methanol (1:1, vol/vol), dried under nitrogen, and redissolved in 5 mL of chloroform. The sample is applied to a silica gel 60 column (1 × 25 cm) equilibrated with chloroform. The column is eluted (approximately 1 mL/min) successively with 100 mL mixtures of chloroform and methanol (from 4 to 14% methanol in 2% increments). Excess *N*-succinimidyl fatty acid ester elutes from the column in the 4% methanol fraction. Lac-Cer elution is monitored in an aliquot of each fraction by HPTLC, and the fractions that contain LacCer are combined.

Separation of individual molecular species of LacCer. Underivatized LacCer was separated (6) into individual molecular species by high-performance liquid chromatography (HPLC) on a C18 reversed-phase (5 μm, Ultrasphere ODS; Beckman, San Ramon, CA) column (4.6 × 250 mm) using a mobile phase of metha-nol/water (96:4, vol/vol) at 30°C at a flow rate varying from 1 mL/min to 1.5 mL/min depending on the carbon chain length of the reacylated fatty acid (the longer the carbon chain, the faster the flow rate) with the effluent being monitored at 205 nm. Each fraction is collected, dried under nitrogen, and Folch partitioned. The lower phase containing LacCer is used for the molecular species identification.

Identification of LacCer molecular species. The individual molecular species of LacCer isolated by HPLC are converted to *O*- and *N*-benzoyl derivatives by reaction with benzoylchloride in pyridine (18) and identified by HPLC on a C18 reversed-phase (3 μm, Spherisorb ODS-2; Metachem Technologies, Redondo Beach, CA) column (2 × 250 mm) using a mobile phase of acetonitrile/2-propanol (8:2, vol/vol) at 30°C at a flow rate of 0.3 mL/min with the effluent being monitored at 230 nm. Peaks are identified by their retention times relative to those of known molecular species of perbenzoylated LacCer (d18:1-16:0, d18:1-18:0, d18:1-24:0 and d20:1-18:0) used as standards, and also by HPLC–mass spectrometry.

Quantitation of LacCer. The perbenzoylated LacCer is quantitated by HPLC on a LiChrospher Si 500 (10 μm; EM Science, Cherry Hill, NJ) column (4.6 × 250 mm) using a mobile phase of hexane/dioxane (88:12, vol/vol) at room temperature at a flow rate of 1 mL/min with the effluent being monitored at 230 nm. The perbenzoylated

LacCer species elute between 14 and 26 min and are quantitated by digital integration.

RESULTS AND DISCUSSION

Bovine brain gangliosides contain primarily d18:1 and d20:1 long-chain bases while d18:0 and d20:0 long-chain bases are present as minor components (19,20). Therefore, when LacCer is prepared from brain gangliosides, the ceramide molecular species of the LacCer reflect the original ganglioside molecular species. It was observed that as the gangliosides were repeatedly reprocessed by mild acid hydrolysis, the proportion of d20:1 long-chain base in the resulting LacCer fraction increased from approximately 30 to over 60%. Thus, it appears that glycosphingolipids containing a d18 long-chain base were more readily hydrolyzed under mild acidic conditions than glycosphingolipids containing a d20 long-chain base.

Procedures for deacylating glycosphingolipids have been reported for cerebrosides (16), GM3 (21), GM1 (22,23), GM3 and GM1 (24), and a number of other gangliosides (17). The alkaline hydrolysis procedure for cerebrosides using potassium hydroxide in *n*-butanol/water with refluxing at 125°C for 4 h (16) and for gangliosides using potassium hydroxide in methanol with refluxing at 102°C for 25 h (17) both hydrolyzed LacCer to lysoLacCer equally well. The procedure for GM1 ganglioside using tetramethylammonium hydroxide in *n*-butanol/water with refluxing at 100°C for 13 h (22) resulted in more by-products and lower recovery of lysoLacCer. Because of the shorter reflux time, the procedure developed for cerebrosides by Radin (16) was used to prepare lysoLacCer. However, after the reaction, the potassium hydroxide and the hydrolyzed fatty acids were removed by repeated solvent partitioning. Figure 1A shows the results of alkaline hydrolysis of LacCer as monitored by HPTLC. The amount of the unknown by-product detected above lysoLacCer (lane 3) increased when the lysoLacCer was stored for a few days. A similar observation was made in the HPTLC analysis of psychosine (data not shown). We did not identify the structure of these by-products.

Procedures have been reported for the re-*N*-acylation of long-chain bases (25) and of lysoGM1 (17) using *N*-succinimidyl esters of fatty acids, and of lysoGM1 using free fatty acids (22). For reacylation of lysoLacCer, the methods using *N*-succinimidyl fatty acid esters in tetrahydrofuran (25) or in chloroform/methanol/water/triethylamine (17) resulted in equally high yields. The method using free fatty acids (22) did not give good yields. The method for reacylation developed by Neuenhofer *et al.* (17) was used in the present study because it was simpler to purify the products. The lysoLacCer prepared from brain ganglioside LacCer was reacylated with *N*-succinimidyl 16:0, 18:0, 20:0, 22:0, 24:0, 20:1, 22:1 and 24:1 fatty acid esters. Reacylated lysoLacCer was purified on a silica gel 60 column and separated into individual molecular species by HPLC as described in Experimental Procedures section. As an

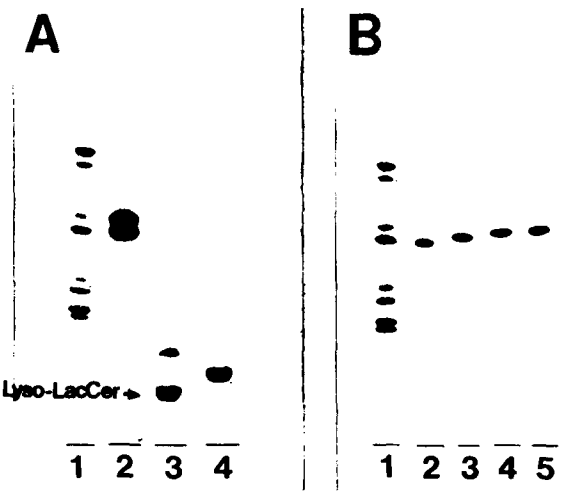

FIG. 1. Thin-layer chromatogram of LacCer and lysoLacCer. LacCer prepared from red blood cell $GbOse_4Cer$ was deacylated as described in Experimental Procedures section. A. Lane 1, neutral glycosphingolipid standards (from top are GlcCer, LacCer, $GbOse_3Cer$ and $GbOse_4Cer$); lane 2, LacCer prepared from $GbOse_4Cer$; lane 3, lysoLacCer; and lane 4, psychosine (lysoGalCer). B. Lane 1, neutral glycosphingolipid standards; lane 2, LacCer deacylated and reacylated with 16:0; lane 3, reacylated with 20:0; lane 4, reacylated with 22:0; lane 5, reacylated with 22:1. High-performance thin-layer chromatography plates were developed in chloroform/methanol/water (60:35:8, by vol) and visualized with orcinol spray and heating at 130°C (14).

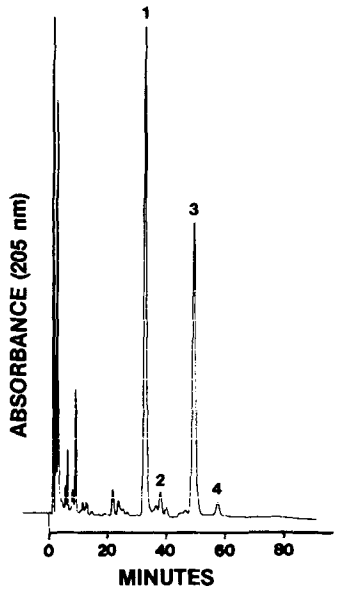

FIG. 2. Molecular species separation of LacCer after deacylation followed by reacylation. LacCer prepared from brain gangliosides was deacylated and then reacylated with *N*-succinimidyl 20:0 fatty acid ester and purified on a silica gel 60 column as described in Experimental Procedures section. Underivatized LacCer was separated into individual molecular species by high-performance liquid chromatography on a C18 reversed-phase column (5 μm, Ultrasphere ODS, 4.6 × 250 mm) at 30°C. The mobile phase was methanol/water (96:4, vol/vol) at a flow rate of 1.2 mL/min. The sample was injected in ethanol. The effluent was monitored at 205 nm with an attenuation of 0.16 absorbance unit full scale. Peak 1, d18:1-20:0; peak 2, d18:0-20:0 (approximately 70%); peak 3, d20:1-20:0; and peak 4, d20:0-20:0.

example, the molecular species pattern of LacCer reacylated with 20:0 fatty acid is presented in Figure 2. The major molecular species of reacylated LacCer were d18:1-20:0 (peak 1) and d20:1-20:0 (peak 3), and the minor molecular species were d18:0-20:0 (peak 2) and d20:0-20:0 (peak 4).

An aliquot of each of the LacCer molecular species was perbenzoylated, and purity was determined by HPLC using an acetonitrile-containing mobile phase. With this mobile phase, it is possible to separate molecular species containing a saturated fatty acid from a molecular species that contains a monounsaturated fatty acid with two more carbons but the same long-chain base, e.g., d18:1-22:0 from d18:1-24:1 (26). Moreover, using a mobile phase of acetonitrile/2-propanol (8:2, vol/vol) and a 3 μm C18 reversed-phase column, perbenzoylated LacCer molecular species which have the same number of carbons and the same number of double bond, e.g., d18:1-20:0 and d20:1-18:0, could also be resolved into two peaks (data not shown). As seen in Figures 3 and 4, all molecular species of LacCer prepared were single molecular species. It appears, however, that lysoLacCer reacylated with unsaturated fatty acids (Fig. 4) are less pure than those reacylated with saturated fatty acids (Fig. 3). For d18:1 and d20:1 long-chain bases, over 95% of the reacylated LacCer were one molecular species. It was difficult to obtain relatively pure molecular species of LacCer containing d18:0 long-chain base coupled with the fatty acid introduced (Fig. 2, peak 2) due to contamination with other minor components. Because of the small amount of these contaminants, we were not able to identify them. On the other hand, the LacCer molecular species containing d20:0 long-chain base coupled with the fatty acid introduced (Fig. 2, peak 4) was relatively pure (approximately 90%). The identity of each molecular species of LacCer was confirmed by HPLC–mass spectrometry (data not shown).

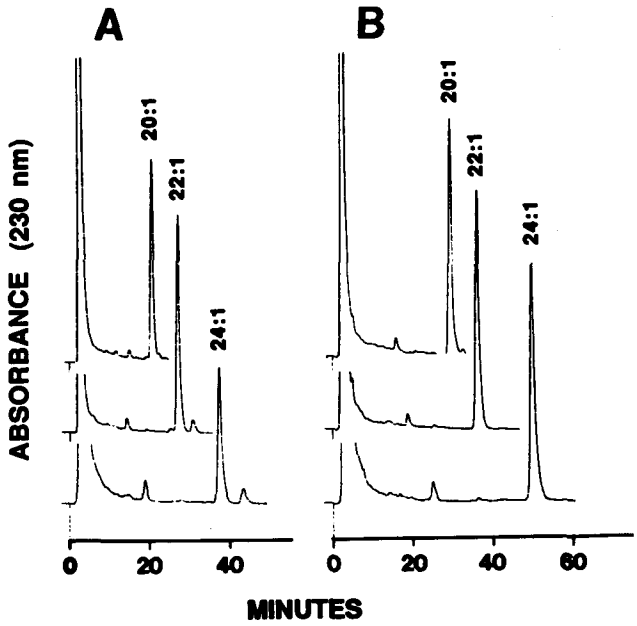

FIG. 4. Determination of the purity of LacCer molecular species reacylated with unsaturated fatty acid. LacCer reacylated with *N*-succinimidyl 20:1, 22:1 or 24:1 fatty acid ester was separated into individual molecular species and the purity was determined by high-performance liquid chromatography as described in Figure 3. A, LacCer containing d18:1 long-chain base; B, LacCer containing d20:1 long-chain base. The fatty acid moiety is identified above each peak.

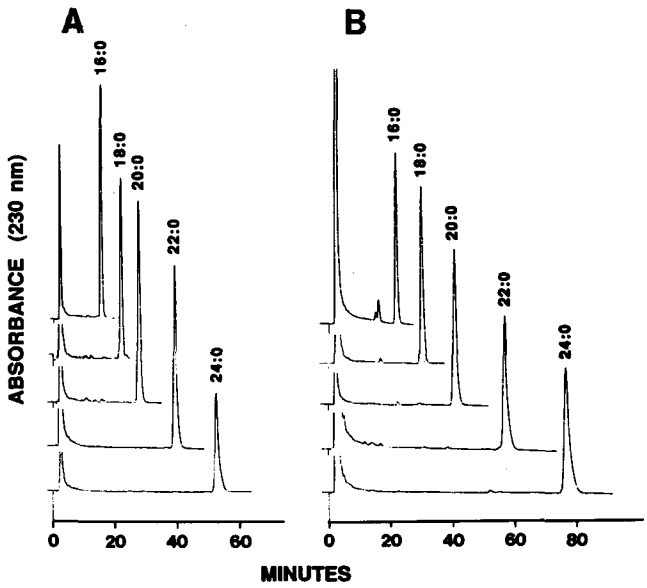

FIG. 3. Determination of the purity of LacCer molecular species reacylated with saturated fatty acid. LacCer prepared from brain gangliosides was deacylated and then reacylated with *N*-succinimidyl 16:0, 18:0, 20:0, 22:0 or 24:0 fatty acid ester, and separated into individual molecular species as shown in Figure 2. Major peaks were collected, perbenzoylated, and their molecular species and purity were determined by high-performance liquid chromatography on a C18 reversed-phase column (3 μm, Spherisorb ODS-2, 2 × 250 mm) at 30°C. The mobile phase was acetonitrile/2-propanol (8:2, vol/vol) at a flow rate of 0.3 mL/min. The sample was injected in the mobile phase. A, LacCer containing d18:1 long-chain base; B, LacCer containing d20:1 long-chain base. The fatty acid moiety is identified above each peak.

The recovery of reacylated lysoLacCer was determined relative to the original LacCer used as starting material. For this determination, the original LacCer was prepared from $GbOse_4Cer$ because the LacCer prepared from red blood cell $GbOse_4Cer$ contains only d18:1 as long-chain base, and that the product LacCer after de/reacylation contains only a single molecular species. In the present study, *N*-succinimidyl 16:0, 20:0, 22:0 or 20:1 fatty acid ester was used for reacylation. Each newly synthesized LacCer showed a single band in HPTLC (Fig. 1B, lanes 2–5) and gave a single peak in HPLC (data not shown). The yields of LacCer relative to the original LacCer (650 nmol) used as starting material upon reaction with *N*-succinimidyl 16:0, 20:0, 22:0 or 22:1 fatty acid ester were 290 nmol (45%), 376 nmol (58%), 277 nmol (43%) and 245 nmol (38%), respectively. Thus, it appears that reacylation with saturated fatty acids results in higher yields.

As seen in Figures 3 and 4, the L-isomer, which can clearly be resolved from the D-isomer by HPLC (data not shown), was not observed. Moreover, as reported by Neuenhofer *et al.* (17), the *threo*-sphingoid configuration

was not formed in the course of the deacylation and reacylation procedures. The methods described here are therefore useful for preparing a variety of pure D-*erythro*-LacCer molecular species.

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COMMUNICATION

Apolipoprotein A-I, A-IV and E Synthesis in the Liver of Copper-Deficient Rats

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Copper deficiency induces hypercholesterolemia in the rat. This hypercholesterolemia is mainly due to an increase in apo E-rich high density lipoproteins (HDL_1). The present study was undertaken to determine whether the HDL increase could be explained by altered low-molecular weight apolipoprotein (apo) synthesis in the liver. The effect of copper deficiency on apo A-I, apo A-IV and apo E concentrations in plasma, as well as on respective mRNA levels and synthesis in the liver, were therefore investigated. We observed that the increased HDL_1 levels in the plasma of copper-deficient rats were associated with a significant rise in plasma apo E concentrations; however, plasma apo A-I and apo A-IV concentrations remained unchanged. Liver apo synthesis and respective apo mRNA levels were not significantly altered in copper-deficient animals when compared to control rats. No changes in apo E mRNA levels in various tissues from copper-deficient, as compared to control rats, were noted. Based on the data obtained, it was concluded that the observed changes in plasma lipoprotein and apo concentrations are not related to changes in low-molecular weight apo synthesis in the liver. The mechanisms of the impaired catabolism of HDL_1 should be further evaluated to possibly explain the observed increase in this fraction in copper-deficient rats.

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Copper deficiency has been shown to induce hypercholesterolemia in humans and in various experimental animals (1,2). It was suggested that hypercholesterolemia in copper-deficient rats may be the result of an increased rate of cholesterol synthesis in the liver (1–3). Recently Kim *et al.* (4) proposed that copper deficiency-induced hypercholesterolemia and elevated 3-hydroxy-3-methyl-glutaryl coenzyme A reductase activity may be due to elevated hepatic glutathione. The authors (4) have shown that inhibition of elevated hepatic glutathione abolishes the increase in plasma cholesterol concentration. Copper deficiency-induced hypercholesterolemia is associated with changes in lipoprotein concentration and composition (2,5,6). We have recently shown that increased apolipoprotein (apo) B synthesis in the liver accompanies copper deficiency induced hyperlipemia and an increase in plasma apo B concentration (7). Moreover, the specific rise in apo B100 synthesis suggested that this increase may contribute to the elevated plasma low density lipoprotein concentrations. The induction of apo B synthesis through copper deficiency may be related to the increased production of fatty acids and cholesterol in the liver for both the assembly and the secretion of triglyceride-rich lipoproteins (2). However, in copper-deficient rats, increases in plasma lipid and apo levels are mostly associated with high density lipoprotein (HDL) (5,6,8). In the present study, we therefore investigated whether the increase in HDL could be explained by changes in low-molecular weight apo synthesis in the liver. We followed the effect of copper deficiency on apo A-I, apo A-IV and apo E concentrations in plasma as well as on the respective mRNA levels and synthesis in the liver.

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Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Weanling male Wistar rats (Iffa-Centre des Recherches et d'Elevage des Oncins, L'Arbresle, France) were randomly divided into copper-deficient and control groups. Rats were housed in wire-bottomed cages in a temperature-controlled room (22°C) with a 12-h light/dark cycle and were pair-fed appropriate diets for six weeks. The semi-purified diets contained (g/kg): casein, 200; sucrose, 650; corn oil, 50; alphacel, 50; DL-methionine, 3; choline bitartrate, 2; modified AIN-76 mineral mix, 35; and AIN 76A vitamin mix, 10 (ICN Biomedicals, Orsay, France). Cupric carbonate was omitted from the AIN-76 mineral mix in the copper-deficient diet. As assessed by atomic spectrophotometric analysis (model 560; Perkin Elmer, Norwalk, CT), the copper concentrations of the two diets were 0.4 mg/kg (deficient) and 6 mg/kg (control). Animals were anesthetized with sodium pentobarbital (40 mg/kg body weight i.p.) and then killed. Blood was collected into tubes containing ethylenediaminetetraacetic acid or heparin, and plasma was obtained by low-speed centrifugation ($2,000 \times g$). Copper in plasma was determined by atomic absorption analysis. Total cholesterol (BioMérieux, Charbonnières-les-Bains, France) in plasma was determined enzymatically. Apo were determined by radial immunodiffusion using sheep anti-rat apo antisera; plasma samples were diluted with Nonidet P40 (0.5% final concentration) for apo E assay and with urea (7M final concentration) for apo A-I and apo A-IV assays (9,10).

Lipoprotein fractions were separated from plasma by continuous gradient ultracentrifugation in an L8.70 Beckman (Beckman Instruments, Palo Alto, CA) centrifuge using a SW 41 rotor as described previously (11). Twenty-two fractions were collected from the meniscus of the tubes, and total cholesterol content in these fractions was assayed.

Total cell RNA was isolated from tissues using the guanidinium/phenol/chloroform method described by Chomczynski and Sacchi (12). Northern and dot blots of total cellular RNA, labeling of probes, hybridization and washing of filters were done as described previously (13,14). The cDNA probe for rat apo E (kindly provided by P. Cardot, URA-CNRS 1283, Paris, France) and oligonucleotide probes for apo A-I and apo A-IV were used to de-

tect specific mRNA (13,14). A mouse 18S rRNA cDNA clone was used as control probe (kindly provided by G. Veyssière, Université Blaise Pascal, Clermont Ferrand, France). Quantification of specific mRNA was performed on autoradiograms of filters by densitometric analysis using a laser densitometer (Ultroscan XL; LKB, Bromma, Sweden), and values were expressed in absorbance units relative to the level for control animals.

Apo synthesis rates were determined in the liver of copper-adequate and copper-deficient rats after intraportal vein injection of 1.0 mCi L-[4,5-^{3}H]leucine (specific activity 5.29 TBq/mmol; Amersham, Buckinghamshire, United Kingdom), as described by Baum *et al.* (15). Quantitative immunoprecipitation of apo was done on liver homogenates using polyclonal rabbit antisera directed against rat apo E, A-I and A-IV. The immune complexes obtained were washed extensively and separated on denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis disc gels (7.5% acrylamide). Gels were sliced and radioactivity was determined by liquid scintillation spectrometry (Kontron, St-Quentin-en-Yvelines, France) following the addition of 3% Protosol-Econofluor (NEN, Boston, MA) to gel slices. Data are expressed as percent of total [trichloroacetic acid (TCA)-insoluble] radioactivity.

Values are given as means ± SEM. Data were analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

As previously noted in other laboratories (1,16), as well as in our own (7,17,18), we observed, in addition to reduced plasma copper concentrations, a number of changes that are usually associated with copper deficiency (Table 1). As shown in Table 1, significantly lower body weights and higher relative heart and liver weights were seen in rats fed the copper-deficient diets when compared to controls. In agreement with previous works (2,17,18), plasma cholesterol concentrations were significantly elevated in copper-deficient rats when compared to those of controls (Table 1). As is shown by density gradient ultracentrifugational analysis of plasma lipoproteins (Fig. 1), hypercholesterolemia is mainly due to an increase in the concentration of HDL, and particularly HDL_1. This is consistent with earlier observations (4,19) when plasma lipoproteins were analyzed by polyacrylamide gradient gel electrophoresis and by gel filtration. In the present study, we have clearly shown that the changes in lipoprotein distribution in copper-deficient rats are associated with an increase in plasma apo E concentration. However, copper deficiency had no significant effect on plasma apo A-I and apo A-IV levels (Table 2).

Studies on apo A-I, A-IV and apo E synthesis in the liver have shown (Table 2) that the synthesis of these apo is not significantly altered by copper deficiency. Total protein

TABLE 1

Effect of Copper Deficiency on Body Weight, Relative Organ Weights and Plasma Copper and Cholesterol Concentrations[a]

	Control	Copper-deficient
Body weight (g)	289 ± 3	242 ± 8[b]
Relative liver weight (g/100 g)	3.8 ± 0.1	6.1 ± 0.2[b]
Relative heart weight (g/100 g)	0.33 ± 0.01	0.73 ± 0.05[b]
Plasma copper (μmol/L)	14.60 ± 0.50	0.60 ± 0.08[b]
Plasma total cholesterol (mmol/L)	1.55 ± 0.06	1.98 ± 0.11[b]

[a]Means of 12 animals per group ± SEM.
[b]Significant difference from control rats, $P < 0.001$.

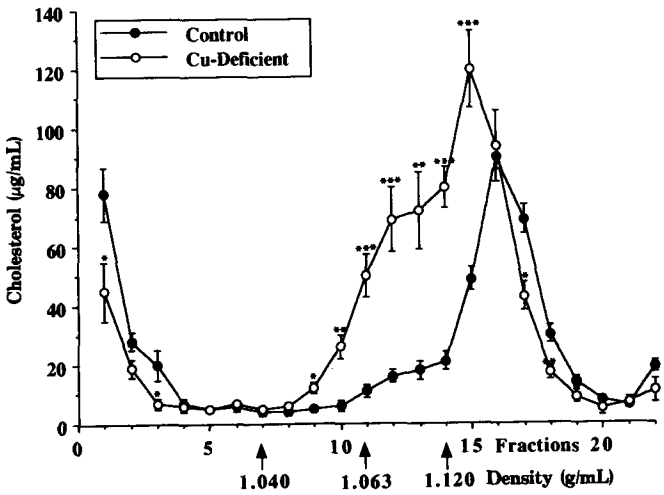

FIG. 1. Effect of copper deficiency on cholesterol distribution in lipoprotein fractions separated by density gradient ultracentrifugation. The results represent the mean (± SEM) of six determinations for control and of five determinations for copper-deficient rats. The points that were significantly different between control and copper-deficient rats are designated with an asterisk (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

TABLE 2

Effect of Copper Deficiency on Apolipoprotein A-I, A-IV and E Concentrations in the Plasma and on Their Synthesis and mRNA Levels in the Liver[a]

	Control	Copper-deficient
Apo A-I		
Plasma (mg/100 mL)	27.8 ± 1.7	29.1 ± 1.8
Synthesis in liver (% of TCA-insoluble radioactivity)	0.19 ± 0.02	0.16 ± 0.02
Liver mRNA (AU)	100 ± 5	93 ± 3
Apo A-IV		
Plasma (mg/100 mL)	16.6 ± 0.7	17.5 ± 1.0
Synthesis in liver (% of TCA-insoluble radioactivity)	0.21 ± 0.03	0.18 ± 0.03
Liver mRNA (AU)	100 ± 6	87 ± 2
Apo E		
Plasma (mg/100 mL)	17.5 ± 0.3	23.2 ± 0.8[b]
Synthesis in liver (% of TCA-insoluble radioactivity)	0.98 ± 0.03	0.88 ± 0.17
Liver mRNA (AU)	100 ± 7	96 ± 7

[a]Means of 12 animals per group ± SEM for plasma apolipoproteins, and of six animals ± SEM per group for apolipoprotein mRNA levels and synthesis. TCA, trichloroacetic acid; AU, absorbance unit.
[b]Significant difference from control rats, $P < 0.001$.

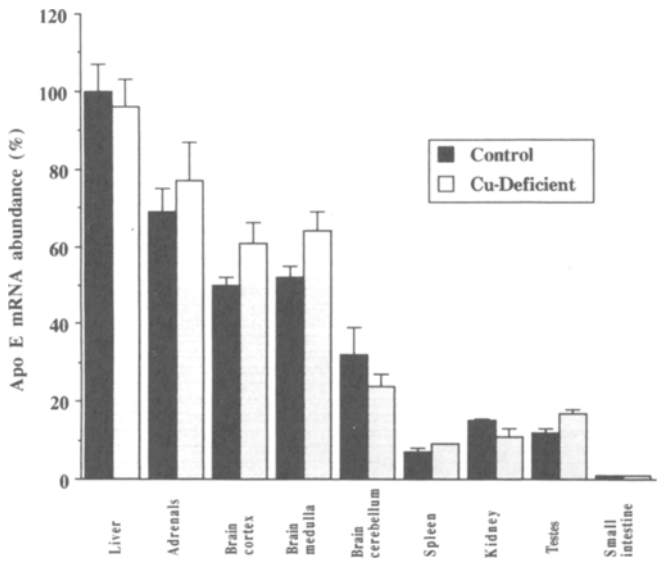

FIG. 2. Effect of copper deficiency on apolipoprotein (apo) E mRNA abundance in various tissues. The amount of apo E mRNA in each tissue is expressed relative to the amount in the liver of control rats (taken as 100%). Values are mean ± SEM of six rats per groups. There was no significant difference ($P > 0.05$) between both groups.

synthesis in the liver, as indicated by [^{3}H]leucine incorporation into TCA-insoluble material, was similar between both groups (control, 118 ± 10 cpm/μg protein; copper deficient, 110 ± 13 cpm/μg protein; $P > 0.05$). Copper deficiency did not significantly change apo A-I, apo A-IV and apo E mRNA levels in the liver either (Table 2). As apo E is synthesized not only by hepatocytes but by a wide variety of mammalian cells (20), we studied the effect of copper deficiency on apo E mRNA levels in several tissues from control and copper-deficient rats. Consistent with results of previous studies done in the rat (20), apo E mRNA was most abundant in the liver, but substantial amounts of apo mRNA were also found in adrenals and in brain (Fig. 2). Copper deficiency did not significantly alter the apo E mRNA levels in all tissues studied (Fig. 2).

In summary, one can conclude from the present work that copper-deficiency does not affect apo E, apo A-I and apo A-IV synthesis in the liver. However, since the liver weight and, in particular, the relative liver weight increase in copper-deficient rats, the possibility exists that the increase in liver mass may contribute to the apparent absolute increase in apo synthesis by this organ. On the other hand, the increase in plasma volume in copper-deficient animals may have an effect on plasma apo concentration independent of changes in apo metabolism. This will need to be considered in future work. Furthermore, as the specific increase in apo E plasma concentration, without changes in apo A-I and apo A-IV concentrations, occurs in copper deficiency, the mechanisms of the impaired catabolism of HDL_1 should be evaluated to pinpoint the cause of the increase in this lipoprotein fraction.

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Alkyl Glycerol Monoethers in the Marine Sponge *Desmapsamma anchorata*[1]

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1-*O*-Hexadecylglycerol (chimyl alcohol), 1-*O*-heptadecylglycerol and 1-*O*-octadecylglycerol (batyl alcohol) have been identified as the major native constituents of a mixture of free alkyl glycerol ethers isolated from the contained water and the methanolic extract of the sponge *Desmapsamma anchorata*. Minor components were the free C_{14}, C_{15}, C_{19}, C_{20} and C_{21} alkyl glycerol monoethers. The alkyl glycerol monoethers were analyzed and identified by gas chromatography/mass spectrometry of their isopropylidene derivatives. This is the first report on the occurrence of free C_{15}, C_{19}, C_{20} and C_{21} alkyl glycerol monoethers in a sponge.
***Lipids* 29, 731–734 (1994).**

The saturated monoalkyl ethers of glycerol, 1-*O*-hexadecyl-*sn*-glycerol(**1c**; chimyl alcohol) (see Fig. 1); and 1-*O*-octadecyl-*sn*-glycerol(**1e**; batyl alcohol) commonly occur esterified to fatty acids in a wide variety of marine organisms (1–5). They have also been found in some marine sponges in the unesterified form (6–11). Thus, free chimyl alcohol **1c** was recently found in *Stylopus australis* (Poecilosclerida:Hymedesmiidae) (6), *Tethya aurantia* (Hadromerida:Tethyidae) (7) and *Tedania ignis* (Poecilosclerida:Myxillidae) (8), while batyl alcohol **1e** was found in *Ulosa ruetzleri* (Axinellida:Axinellidae) (9) and *T. ignis* (8). The first report on the occurrence of a free alkyl glycerol monoether in a sponge was that on 1-*O*-tridecyl-*sn*-glycerol (**1i**) from a sponge of the family Plocamiidae (10). At about the same time, the branched-chain alkyl glycerol monoesters **5a** and **5b** were isolated from a Taiwanese marine sponge of genus *Aaptos* (Hadromerida:Suberitidae) (11) and **1j** and **1k** from *T. aurantia* (7).

Desmapsamma anchorata (Poecilosclerida:Esperiopsidae) first described by Carter in 1882 (12) and more recently by Green *et al.* (13), is a *Desmapsamma* ramose sponge erected on an encrusting base. Alive, it is pale pink externally and dark orange internally, and it is smooth and "spongy." *Desmapsamma anchorata* inhabits its hexacoralia plates and *Acropora* cemeteries on the leeward side of coral reefs in the Gulf of Mexico and Caribbean Sea. It has previously been shown that the Caribbean *D. anchorata* contains interesting fatty acids with unusual unsaturation and methyl branching as well as the common C_{14} to C_{26} fatty acids and common sponge sterols; but no free alkyl glycerol ethers were found (14). We report here on the isolation of free saturated monoalkyl ethers of glycerol from *D. anchorata* harvested in the Gulf of Mexico.

[1]Contribution No. 1241 from the Instituto de Química, UNAM. This work was presented in part at the First Lanbio Symposium, Trends in Natural Products Research: Prospects for Pharmacological and Agrochemical Applications, Asunción, Paraguay, August 6–10, 1993.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

EXPERIMENTAL PROCEDURES

Sponge collection. Approximately 30 specimens of *D. anchorata* were collected at depths of 2 to 15 m and at a water surface temperature of 28°C near the La Anegada de Afuera reef (Veracruz, Ver., Mexico) in June 1989. A voucher specimen is being kept at the Laboratorio de Farmacología Marina, Instituto de Ciencias del Mar y Limnología (UNAM). *Desmapsamma anchorata* is a shallow water species which shows no association with symbiotic algae but considerable affinity for *Holopsamma helwigi* at the same location. *Desmapsamma anchorata* is a very abundant species near the reefs of Veracruz, Mexico.

Isolation. The sponge (6.0 kg) was squeezed, and the water from the sponge was filtered through a Büchner funnel using a celite layer as filter aid to remove insoluble materials. The filtrate was extracted with ethyl acetate (4 × 500 mL). The insoluble material was washed from the celite layer first with petroleum ether (4 × 2 L) and then with methanol (4 × 2 L). The ethyl acetate extract and the petroleum ether and methanol washings were taken to dryness *in vacuo* to yield 1.42, 0.43 and 0.94 g of material, respectively. The residues were then combined as they were found to be similar as judged by thin-layer chromatography (TLC) using the developing solvent dichloromethane/acetone (9:1, vol/vol).

To the remaining sponge material (545.5 g dry wt), methanol (5 × 2.5 L) was added, and the mixture was filtered through celite. The aqueous methanol phase was brought to dryness and then extracted with dichloromethane and ethyl acetate (4 × 2 L each). The organic extracts were concentrated *in vacuo* to yield a reddish-brown oil (35 g). The residue from the aqueous phase of the sponge was chromatographed on a column (4.5 × 35.0 cm) of 60 g of silica gel, while the residue from the remaining sponge material was separated on a column (6.0 × 35.0 cm) containing 170 g of silica gel, and the columns were eluted with petroleum ether, petroleum ether/ethyl acetate mixtures, and methanol. Elution with petroleum ether yielded 135 mg of a mixture of fatty acid methyl esters which were likely formed during methanol extraction. Gas chromatography/mass spectrometry (GC/MS) analysis of the mixture indicated the presence of C_{14} to C_{21} fatty acids which had previously been identified in this species (14). Petroleum ether/ethyl acetate (9:1, vol/vol) yielded 4.2 g of a mixture of sterols (m.p. 134–140°C). Acetylation of the

sterol mixture (60 mg) gave the corresponding acetates. GC/MS of the acetates confirmed the presence of the sterols previously identified in this species (14), i.e., desmosterol (3.9%), cholesta-5,22-dienol (9.2%), cholesterol (44.6%), 24-methylcholesta-5,22-dienol (18.3%), 24-methylcholesterol (6.2%), stigmasterol (4.4%) and sitosterol (11.1%). Elution with petroleum ether/ethyl acetate (4:1 and 3:1, vol/vol) yielded a mixture of the monoalkyl ethers of glycerol (10 mg from aqueous phase, and 300 mg from sponge) as an amorphous solid (**1a–h**). The compounds found in the aqueous and sponge residues were similar.

Optical rotations were measured on $CHCl_3$ solutions using a Jasco DIP-360 Digital polarimeter (Japan Spectroscopic Co., Tokyo, Japan). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra on $CDCl_3$ solutions, with tetramethylsilane (TMS) as internal standard, were recorded at 300 MHz or 200 MHz, either on a Varian VXR-300S instrument or a Varian Gemini-200 instrument (Varian, Palo Alto, CA). Mass spectra (MS) were recorded using a Hewlett-Packard MS5985B mass spectrometer (Palo Alto, CA). GC/MS was done using a PAS-1701 (ECD tested 1701 silicone) column (25 × 0.32 mm × 0.25 μm) in a Hewlett-Packard 5890 Series II gas chromatograph linked to a JEOL JMS-AX505HA mass spectrometer with a data system (Tokyo, Japan). Samples were analyzed using temperature programming: first isothermal at 100°C for 1 min, then increased at 10°C/min to 280°C, and finally kept at 280°C for 10 min. TLC was done on silica gel plates impregnated with fluorescent dye (Merck Silica Gel 60 F_{254}; Merck, Darmstadt, Germany).

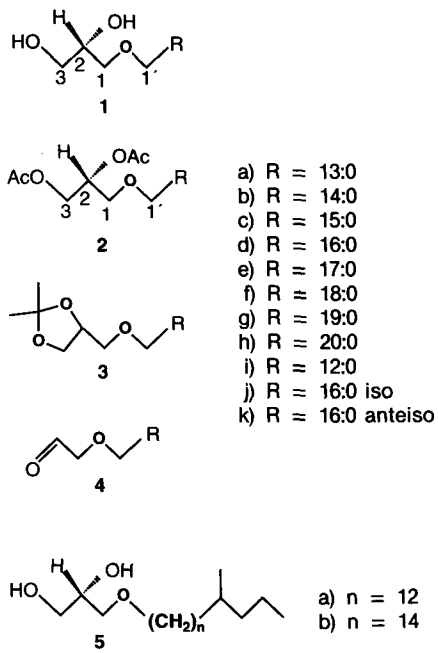

FIG. 1. Structures of alkyl glycerol monoethers (1a–h) from *Desmapsamma anchorata* and of some structural analogs (2a–h, 3a–h) as well as of some related compounds from other sponges (1i–k, 5a–b).

*Monoalkyl ethers of glycerol (Fig. 1, **1a–h**).* ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 *t*, *J* = 6.7 Hz, CH_3; 1.26, *s*, $(CH_2)_n$, aliphatic chain; 1.57, *quint*, *J* = 6.9 Hz, H-2′; 3.46, AB *m*; *apparent td*, *J* = 6.6 Hz, *J* = 1.2 Hz, H-1′; 3.48, *dd*, *J* = 9.6 Hz, *J* = 6.0 Hz, H-1a; 3.53, *dd*, *J* = 9.6 Hz, *J* = 4.2 Hz, H-1b; 3.63, *dd*, *J* = 11.7 Hz, *J* = 5.4 Hz, H-3a; 3.71, *dd*, *J* = 11.7 Hz, *J* = 3.9 Hz, H-3b; and 3.86, *pseudo quint*, *J* = 5.4 Hz, H-2. ^{13}C NMR δ ($CDCl_3$, 75 MHz) δ 14.13, 22.70, 26.08, 29.36, 29.47, 29.58, 29.59, 29.62, 29.67, 29.70, 31.92, 64.23, 70.48, 71.83, 72.43. The chemical shifts for H-1 and H-3 are reverse compared to those described in Ref. 15. Assignments for H-2 and H-3 were confirmed by the downfield shifts of the signals in the ^1H NMR spectrum of the acetylation product.

Acetylation of the monoalkyl ethers of glycerol. An aliquot of monoalkyl ethers (10 mg) was treated with Ac_2O (0.2 mL) and pyridine (0.1 mL) at room temperature overnight. The excess reactants were removed on a rotary evaporator under reduced pressure. The residue was chromatographed on a column of silica gel to yield the diacetates (Fig. 1, **2a–h**) (5 mg) as a yellowish oil. ^1H NMR ($CDCl_3$, 300 MHz) δ 0.89, *t*, CH_3; 1.26, *s*, CH_2, aliphatic chain; 1.56, *quint*, H-2′; 2.07, *s*, AcO; 2.10, *s*, AcO; 3.44, AB *m*, H-1′; 3.56, *d*, *J* = 5.3 Hz, H-1; 4.16, *dd*, *J*= 11.5 Hz, *J* = 5.8 Hz, H-3b; 4.34, *dd*, *J* = 11.5 Hz, *J* = 4.0 Hz, H-3a; and 5.19, *m*, H-2. ^{13}C NMR δ ($CDCl_3$, 75 MHz) 14.15, 20.81, 21.08, 22.71, 26.03, 29.37, 29.46, 29.53, 29.62, 29.64, 29.72, 31.94, 62.99, 68.82, 70.31, 71.76.

Isopropylidene derivatives of monoalkyl ethers of glycerol. To a solution of monoalkyl ethers of glycerol (20 mg) in dry acetone (12 mL), dry $CuSO_4$ was added in excess and refluxed with stirring for 2 h. The reaction was monitored by TLC. The mixture was filtered, and the filtrate was directly subjected to silica gel column chromatography. Elution with petroleum ether/CH_2Cl_2 (4:1, vol/vol) yielded the isopropylidene derivatives (Fig. 1, **3a–h**). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88, *t*, CH_3; 1.26, *s*, CH_2, aliphatic chain; 1.37, *s*, 3H, 1.43, *s*, 3H, gem dimethyl; 1.56, *quint*, H-2′; 3.41 and 3.52, *dd*, *J* = 9.8 Hz, *J* = 5.6 Hz, H-1b and H-1a; 3.46, AB *m*, H-1′a and H-1′b; 3.73 and 4.07, *dd*, *J* = 8.2 Hz, *J* = 6.4 Hz, H-3b and H-3a; and 4.28, *pseudo quint*, *J* = 6.2 Hz, H-2. ^{13}C NMR ($CDCl_3$, 175 MHz) δ 14.07, 22.65, 25.38, 26.01, 26.73, 29.33, 29.43, 29.56, 29.65, 29.99, 31.89, 66.93, 71.81, 71.89, 74.75, 109.37.

Periodate oxidation of the monoalkyl ethers of glycerol. A solution of monoalkyl ethers of glycerol (50 mg) in diethyl ether was treated with 38% periodic acid (10 mL) at room temperature overnight, and the reaction mixture was purified by column chromatography to yield the respective glycolaldehyde ethers (Fig. 1, **4a–h**) (10 mg). ^1H NMR ($CDCl_3$, 200 MHz) δ 0.9, *t*, CH_3, *J* = 7.0 Hz; 1.25, *s*, CH_2, aliphatic chain; 1.57, *m*, H-2′; 3.53 *t*, *J* = 6.5 Hz, H-1′; 4.05, *d*, *J* = 1.0 Hz, H-1; and 9.74, *t*, *J* = 1.0 Hz, H-2.

RESULTS AND DISCUSSION

The monoalkyl ethers of glycerol were isolated as a waxy solid (m.p. 57–58°C; $[\alpha]_D$ + 2.0° at c = 0.2 in $CHCl_3$) and

were identified by ^1H NMR and GC/MS of the isopropylidene derivatives. The ^1H NMR spectrum exhibited signals for an aliphatic long chain and signals associated with the presence of the glycerol monoether moiety. Three methylenes and one methine were observed attached to oxygen atoms: a two proton AB multiplet (an apparent triplet of doublets, J = 6.6 Hz, J = 1.2 Hz H-1′) at 3.46 ppm, two AB doublets of doublets centered at 3.48 ppm (J = 9.6 Hz, J = 6.0 Hz, H-1a) and 3.53 ppm (J = 9.6 Hz, J = 4.2 Hz, H-1b), two AB doublets of doublets centered at 3.63 ppm (J = 11.7 Hz, J = 5.4 Hz, H-3a) and 3.71 ppm (J = 11.7 Hz, J = 3.9 Hz, H-3b) and a pseudo quintuplet at 3.86 ppm ($J \approx 5.4$ Hz) for the methine proton (H-2). The ^{13}C NMR spectrum was in agreement with this structure and showed three methylene carbons at 72.43 (C-1), 71.83 (C-1′) and 64.23 (C-3), and a methine at 70.48 (C-2). These assignments are based on multipulse APT and 2D ^1H-^{13}C correlation experiments.

Proton assignments were confirmed by converting the diols to the corresponding diacetates. The ^1H NMR showed two sharp singlets at 2.07 and 2.10 ppm corresponding to the acetyl protons, as well as a downfield shift of the two H-3 doublets of doublets (4.16, J = 11.5 Hz, J = 5.8 Hz, H-3a; 4.34, J = 11.5 Hz, J = 4.0 Hz, H-3b) and the methine proton (H-2, 5.19 ppm).

Oxidative cleavage of the diols with periodic acid gave the corresponding glycoaldehyde alkyl ethers confirming the presence of a 1,2-diol group. The ^1H NMR spectrum exhibited the aldehydic proton at 9.74 ppm as a triplet (J = 1.0 Hz) coupled with the methylene doublet at 4.05 ppm (J = 1.0 Hz).

The above assignments are in good agreement with those published for chimyl and batyl alcohol (**1c, 1e**) as well as other monoalkyl ethers of glycerol **1j, 1k** (7,15,16).

Although useful anomalous ion peaks at one mass unit above the molecular weight [M + 1] have been reported to occur in the spectra of various ethers, alcohols, glycols, amines and nitriles (17), the presence of such a peak has not been reported previously for alkylglycerol ethers (6–11,15). We noted that the electron impact mass spectrum measured at 70 eV on the mixture of monoalkyl ethers of glycerol isolated from *D. anchorata* showed a useful [M + 1] peak at *m/z* 317 (0.9%) and other significant peaks at *m/z* 285 $[M - CH_3O]^+$ (0.6%), 255 $[M - C_2H_5O_2]^+$ (0.9%) and 225 $[M - C_3H_7O_3]^+$ (3.1%) characteristic of chimyl alcohol **1c** as the major component. The mass spectrum also showed additional weak [M + 1] ion peaks at *m/z* 331 (0.2%) and 345 (0.1%) indicative of the presence of C_{17} and C_{18} analogs. Similar results were obtained at 12 eV and in the chemical ionization mode (data not shown).

While mass spectrometry using the direct insertion mode, of the intact alkyl glycerol monoethers indicated the presence of glycerol ethers with 16:0, 17:0 and 18:0 hydrocarbon chains as major constituents, GC/MS of the isopropylidene derivatives identified glycerol ethers with hydrocarbon chain lengths from C_{14} to C_{21}. GC/MS of the isopropylidene derivatives did not give molecular ion peaks, but it showed the $[M - 15]^+$ ion and the characteristic base peak at *m/z* 101 (18). The major saturated 1-*O*-alkyl glycerol ethers in decreasing order of abundance were: C_{16} (56.48%), C_{17} (21.98%), and C_{18} (17.24%), followed by C_{14} (1.65%), C_{15} (1.44%), C_{19} (0.77%), C_{20} (<0.5%) and C_{21} (<0.5%). GC/MS furthermore indicated the presence of two C_{16}, four C_{17}, three C_{18} and two C_{19} saturated alkyl isomers, suggesting the presence of iso, anteiso, and/or other branched structures. It was not possible to identify the position of the methyl branching in the hydrocarbon side chain. It should be noted that GC/MS of the methyl esters of the fatty acids isolated from this sponge also indicated the presence of carbon chain lengths from C_{14} to C_{21} and the presence of saturated branched isomers (14).

Sponges such as *D. anchorata* have been shown to be a good source of phospholipids with fatty acids having unusual unsaturation and methyl branching which is typically not found in terrestrial systems (14). Chimyl alcohol (**1c**), batyl alcohol (**1e**) and their unsaturated analogs are widely distributed in nature and have been found in elasmobranch fish, human milk and bone marrow, atherosclerotic human aorta, pig spleen, in the fat of neonatal calves, in colostrum and milk of cows, goats, pigs and sheep (9), and more recently in reptile skin gland secretions (18). Unesterified alkyl glycerol monoethers have been isolated from marine sponges, although screening of several marine sponges of the class Demospongiae has shown that this type of ether lipid is not widely distributed among sponges (7). Studies on the antimicrobial activity of alkyl glycerol ethers have shown that these compounds inhibit bacterial growth (19). Their role in sponges is still uncertain, although it has been proposed that alkyl glycerol monoethers may play some part in the defense mechanism in the life of the sponge (7).

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ARTICLE

HDL_3-Mediated Cholesterol Efflux from Cultured Enterocytes: The Role of Apoproteins A-I and A-II

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High density lipoproteins (HDL) were recently demonstrated in an enterocyte model (CaCo-2 cells) to mediate reverse cholesterol transport by retroendocytosis. The present study was carried out to define the role of the major HDL apoproteins (apo) A-I and apo A-II in this pathway. HDL_3 was fractionated by heparin affinity chromatography into the two main fractions containing either apo A-I only (fraction A) or both apo A-I and apo A-II (fraction B). In addition, liposomes were reconstituted from purified apo A-I or apo A-II and dimyristoyl phosphatidylcholine. The cell binding properties and cholesterol efflux potential were studied in the lipoprotein fractions and the liposomes. Both fractions exhibited similar maximal binding capacities of 4427 (A) and 5041 (B) ng/mg cell protein, but their dissociation constants differed (40.5 and 167.7 μg/mL, respectively). Fraction A induced cholesterol efflux and stimulated cholesterol synthesis more than did fraction B. Fraction A mobilized both cellular free and esterified cholesterol, whereas fraction B preferentially mobilized cholesteryl esters. Liposomes, containing either apo A-I or apo A-II, showed specific binding, endocytosis and endosomal transport, and were released as intact particles. Apo A-I liposomes also mediated cholesterol efflux. In conclusion, there is evidence that the HDL_3 subfractions A and B, as well as reconstituted liposomes containing either apo A-I or apo A-II, were specifically bound and entered a retroendocytosis pathway which was directly linked to cholesterol efflux. Quantitatively, the apo A-I subfraction appeared to play the dominant role in normal enterocytes. The apo A-II content of fraction B was related to the mobilization of cholesteryl esters.

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Current understanding of cholesterol transport *in vivo* suggests a primary role for high density lipoproteins (HDL) in the reverse cholesterol transport (1) from peripheral tissues to the liver (2). HDL represent a series of dynamically changing HDL populations differing in density, size and composition (3). HDL contains various apolipoproteins (4), most importantly apoprotein A-I (apo A-I) and apoprotein A-II (apo A-II). Specific binding of HDL_3 has been observed in a variety of different cell systems (5–10), as well as in freshly isolated or cultured intestine-derived cells (11–17). Two principally different cellular pathways were suggested for HDL_3-mediated cholesterol efflux: (i) a docking receptor promoting cholesterol translocation (5,18), or (ii) a receptor-mediated intracellular endosomal pathway termed "retroendocytosis" (6,17,19). Both are associated with subsequent efflux of predominantly intracellular as opposed to plasma membrane cholesterol (20). Therefore, a compartmentation of structural and kinetic domains of cholesterol (21–23), as well as a role of the functional intracellular compartmentation of enterocyte cholesterol (24–28), seems feasible. Furthermore, the identification of HDL binding proteins (29–32) has made some progress, but the exact receptor structure is still unclear.

Previous work suggested a different efflux potential of HDL_3 fractions, dependent on the molar ratio of the major apoproteins, apo A-I to apo A-II. In several studies it was shown that apo A-I predominantly promoted cholesterol efflux from adipose cells (33,34) and macrophages (35). In contrast, similar effects of apo A-I and apo A-I/apo A-II particles of different ratios were observed not only in another macrophage system (36), but also in rat hepatoma cells, normal human skin fibroblasts and rabbit aortic smooth muscle cells (37). In bovine aortic endothelial cells (38), the binding affinity and capacity were found to be similar in apo A-I HDL_3 and apo A-II HDL_3. In another study, the particles containing only apo A-I were bound to the membranes of these cells with higher capacity and lower affinity (39). Specific binding and mediation of cholesterol efflux by apo A-IV-containing particles was also recently shown (40). These particles also bind to the apo A-I/apo A-II receptor site of adipose cells (40).

We previously reported that HDL_3 mediates cholesterol efflux from intestinal cells by a retroendocytosis pathway (16,17). The present work defines the role of HDL_3 subfractions containing either apo A-I or apo A-I and apo A-II, as well as the role of reconstituted liposomes of apo A-I or apo A-II, using a previously described culture system of differentiating intestinal cells (17). These cells, derived from a adenocarcinoma of the colon (CaCo-2), are regarded as a valid enterocyte model (41).

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Abbreviations: ACAT, acyl-CoA:cholesterol acyl transferase; apo A-I, apoprotein A-I; apo A-II, apoprotein A-II; BCA, bicinchoninic acid; B_{max}, maximal binding capacity; CaCo-2, carcinoma of the colon cells; FCS, fetal calf serum; DMEM, Dulbecco's minimal essential medium; DMPC, dimyristoyl phosphatidylcholine; HDL, high density lipoproteins; HMG-CoA, hydroxymethylglutaryl coenzyme A; K_D, dissociation constant; LDL, low density lipoproteins; Lip A-I, A-I liposomes; Lip A-II, A-II liposomes; LPD, lipoprotein deficient; MVL, mevalonolactone; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecylsulfate.

MATERIALS AND METHODS

Cell culture system. CaCo-2 cells were obtained from the American Type Culture collection (HTB 37, Rockville, MD) (42). Cells were subcultured and then grown as monolayers on 30-mm Millicell HA filters (Millipore GmbH, Eschborn, Germany) as described previously (17,43). Cells were maintained in 10% (vol/vol) Dulbecco's minimal essential medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with L-glutamine (2 mmol/L), 1% nonessential amino acids, 1% Pen Strep (Gibco) and 20% (vol/vol) fetal calf serum (FCS) (Biochrom, Berlin, Germany) in an atmosphere containing 8% CO_2. Depending on the experimental design, FCS was removed and DMEM was supplemented either with 10% lipoprotein-deficient (LPD) FCS or with fatty acid-free albumin (Sigma Chemicals, Deisenhofen, Germany) as detailed later.

The cells displayed polarized morphology characterized by apical microvilli and a basolateral membrane covering the filter membranes. Confluence of the cell layers was observed around day five after plating. Although the cells partially differentiated during the initial rapid proliferation, a burst of differentiation was primarily observed after day 11 of culture (17). Confluence was observed by light microscopy as well as in a series of electron microscopic studies. Experiments were mainly performed in the postconfluent proliferating phase of cells. Gold-labeled and [^{125}I]labeled HDL_3 fractions and liposomes were exclusively added to the basolateral medium compartment. For efflux measurements, designed with the label [^{14}C]mevalonolactone (MVL) or [^{14}C]octanoate, the fractions and particles used were present basolaterally.

Lipoprotein preparation and isolation. Human plasma was obtained from healthy blood donors. Plasma lipoprotein fractions were separated by ultracentrifugation as described earlier (44). Using potassium bromide for density adjustment, the fractions were isolated at the respective densities of d < 1.006 g/mL for very low density lipoproteins, d 1.019–1.063 for low density lipoproteins (LDL), d 1.063–1.130 for HDL_2 and d 1.130–1.210 for HDL_3. Apo E was removed from the HDL_3 obtained by heparin-Sepharose (CL-6B; Pharmacia, Uppsala, Sweden) affinity chromatography (45) as checked by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). The fractions were detected as distinct homogeneously shaped peaks. The first and quantitatively most important peak (fraction A) only contained apo A-I. The second peak (fraction B) contained apo A-I, slightly lesser amounts of apo A-II and a trace of apo E. To check the purity and the apolipoprotein pattern of the fractions, the lipoproteins were delipidated and the apo residue was incubated with 0.03% SDS and 125 mM mercaptoethanol solution. Subsequently, SDS–PAGE and Coomasie blue staining were used to identify the apo bands. The protein content of the particles was determined using bicinchoninic acid (BCA)(Sigma) as described in the method by Smith *et al.* (46). The lipoprotein concentrations refer to their protein contents. LPD–FCS was prepared from the bottom layer of unfrozen FCS (obtained from Gibco) ultracentrifuged at a density of 1.210 g/mL.

Isolation of the apo A-I and A-II. HDL_3 separated by ultracentrifugation was subjected to the guanidine procedure (47) and repeated dialysis, using a procedural modification (A. Steinmetz, Marburg, Germany, personal communication). The sample was centrifuged at a KBr density of 1.21 g/mL for 24 h at 50,000 rpm. A separation into two phases, an aqueous bottom layer and a lipid containing top layer, was achieved with this procedure. The top layer contained all the lipids, part of apo A-I and most of apo A-II, and the bottom layer mainly contained apo A-I. Compared to apo A-I, only a small quantity of apo A-II could be isolated. To delipidate the lipid containing layer, the phase was incubated several times with diethyl ether/ethanol (1:3, vol/vol) at -20°C for 24 h. The incubation mixture was centrifuged at 2000 rpm for 10 min, and the turbid, lipid-containing supernatant was removed. Then, top and bottom layers were each mixed with a buffer containing 30 mM Tris and 6 M urea at pH 8, and each other was eluted separately over a diethylaminoethyl sephacel column (Pharmacia) using an NaCl gradient up to 125 mM. Subsequently, the samples were dialyzed against 5 mM ammonium bicarbonate. Finally, apo A-I and apo A-II were shown to be pure by SDS–PAGE, were lyophilized, and then stored at -20°C.

Preparation of dimyristoyl phosphatidylcholine (DMPC) A-I and A-II liposomes (Lip A-I and Lip A-II). The liposomes were prepared as previously described (40). Apo and DMPC (Sigma) were mixed in a molar ratio of 1:150. For 10 mg of apo A-I, 36.6 mg of DMPC in chloroform (-20°C) were dispersed in a glass tube and the apo was added. Next, 850 µL of cholic acid (Sigma) [750 mM in phosphate buffered saline (PBS)] were added, mixed and incubated for 30 min at 24°C. To remove excess cholate, the suspension was dialyzed against PBS (40). The protein content of liposomes was measured as described above (46); the liposome concentrations used refer to their protein content.

Colloidal gold preparation and lipoprotein labeling. Preparation of colloidal gold as well as the labeling procedure were essentially done as previously described (17). According to the method of Frens (48), a particle size of 20–40 nm was used in the studies. For the conjugation to HDL_3 fractions and liposomes, the method originally described for LDL (17,49) was used. Gold labeled liposomes were characterized by negative staining. Similar to earlier studies with HDL_3 (16,17), colloidal gold was used as a control, but no specific binding and no internalization were detected.

[^{125}I]Labeling of HDL_3 fractions and liposomes. Using sodium [^{125}I]iodide (Amersham Buchler GmbH, Braunschweig, Germany), Bilheimer *et al.*'s modification (50) of the iodine monochloride method was utilized, arriving at a final specific radioactivity in the range of 200–500 cpm/ng protein for HDL_3 and 100–300 cpm/ng protein for the liposomes. Studies with iodinated and gold labeled lipoproteins and liposomes were performed as previously described (16,17), with minor modifications. Briefly, the cells were incubated in a medium con-

taining 20% FCS until day five and 100 μg/mL LDL (to enrich the cells with cholesterol) between days three and five. The incubation time for binding was 2 h at 4°C. Then, DMEM medium, containing 2 mg/mL albumin, was added, containing increasing concentrations (10–200 μg lipoprotein protein/mL) of [^{125}I]HDL_3 fractions or [^{125}I]liposomes. Specific binding was determined as the difference between total and nonspecific binding, measured with a 50-fold excess of unlabeled HDL_3.

Studies with [^{14}C]labeling of cellular cholesterol. In this set of experiments, procedures and methods were used identical to those described previously (17). Briefly, cells were cultured in DMEM with 20% FCS until day seven and medium was changed every two days. Between days seven and nine, the culture medium contained DMEM, 10% LPD-FCS and 0.5 mM unlabeled octanoate. Newly synthesized cholesterol was labeled using the precursor [^{14}C]octanoate [Du Pont GmbH (NEN), Dreieich, Germany; specific activity 11,600 dpm/nmol], which was also present in the medium between days seven and nine. The HDL_3 subfractions were added to the medium at day eight for a 24-h period. The labeling procedure was done according to a previously described method (25), which had shown comparable results using both [^{3}H]water or [^{14}C]octanoate. Following the incubation, the cells were chilled on ice, and the medium was collected separately, together with three portions of buffer (PBS + 1 mg/mL albumin). Lipids were extracted separately from cells and medium, and free and esterified cholesterol were isolated by thin-layer chromatography (25). Cholesteryl esters were separated and saponified to remove labeled fatty acids (25). Initially, internal standards were routinely added to cell homogenates and medium, to correct for cholesterol loss. Cholesterol synthesis was calculated from the C_2-flux from [^{14}C]octanoate into cholesterol (51).

In a set of time course experiments, DMEM medium containing 20% FCS was present until day five of culture. For cholesterol loading of the cells, cells were incubated in 5 mM MVL (Sigma) between days four and five of culture. FCS was replaced by 10% LPD-FCS to minimize the availability of exogeneous unlabeled cholesterol and the label [^{14}C]MVL [Du Pont GmbH (NEN)] was added from day five to six. The labeling incubation was carried out at 37°C for 24 h. The specific activity was identical in all subsequent sets of experiments (1.5 μCi/filter, 0.5 μCi/mL). To prevent the esterification of labeled nascent cholesterol, 5 μg/mL of Sandoz-Compound 58035 (gift of Sandoz Research Institute, East Hanover, NJ) was added only during the labeling incubation. The labeling procedure was stopped by chilling the cells on ice. Unspecific membrane-associated radioactivity was removed by intensively washing the cells (PBS + 1mg/mL albumin). Subsequently, the medium was changed to DMEM-supplemented with 1 mg/mL albumin, as well as with the different HDL_3 fractions (present only in the basolateral compartment), and efflux of preformed sterols was measured during a 24-h incubation at 37°C. In aliquots of 200 μL of medium, the cell detritus was removed by centrifugation, and the released radioactivity was determined and calculated per mg cell protein. Cellular protein content was determined as described above (46).

Statistics. For statistical analysis, the Student's *t*-test for paired or unpaired samples was used as applicable (* = $P \leq 0.05$; ** = $P \leq 0.01$).

RESULTS

Binding of [^{125}I]labeled HDL_3 subfractions A and B. The HDL_3 density class represents a mixture of particles differing in their apo pattern. The further separation by heparin–sepharose affinity chromatography revealed two main fractions, alternatively containing apo A-I (fraction A) or apo A-I plus apo A-II (fraction B,) which both were used for further experiments. Specific binding of [^{125}I]labeled HDL_3 fractions A and B was saturable and concentration-dependent (Fig. 1). Compared to fraction A, the apo A-II containing fraction B showed a lower level of specific binding at all concentrations tested (Fig. 1). The maximal binding capacity (B_{max}) for fraction A, as derived from Scatchard analysis of the mean values, amounted to 4427 ng/mg cell protein, and the dissociation constant (K_D) was 40.5 μg/mL (correla-

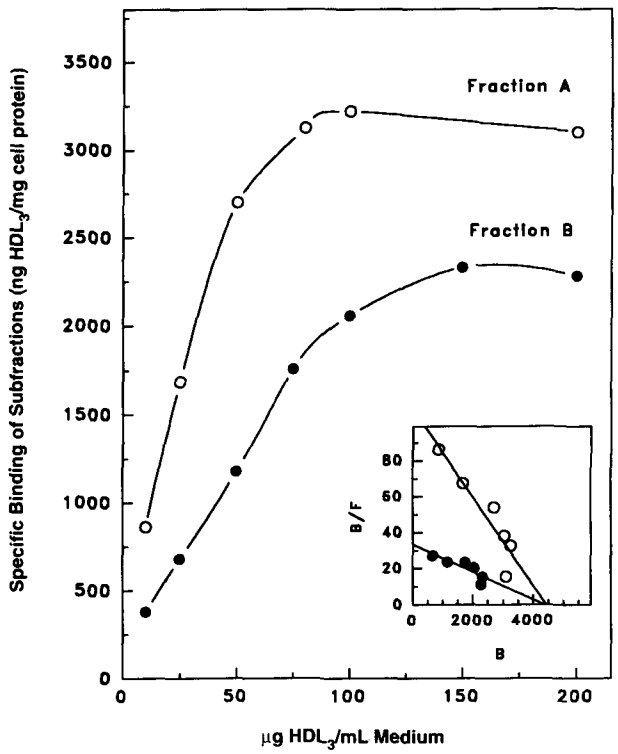

FIG. 1. Binding of HDL_3 subfractions A and B. CaCo-2 cells were cultured with 20% fetal calf serum until day five, 100 μg/mL low density lipoprotein were added from days three to five, and binding was measured at 4°C for 2 h as described in the Materials and Methods section. The curves show specific binding and the corresponding Scatchard analysis, which was calculated from the mean values. The data represent the mean values of two separate experiments (which varied by less than ±8%). Abbreviations: HDL, high density lipoprotein.

tion coefficient r = 0.927). The corresponding values for fraction B were 5041 ng/mg cell protein and 167.7 μg/mL (K_D) (r = 0.832).

Cholesterol synthesis from [^{14}C]octanoate and HDL$_3$ subfraction A- and B-mediated efflux. In this set of experiments, cholesterol synthesis was measured with small amounts of octanoate which did not effectively enhance cellular cholesterol content. The labeling process included prelabeling as well as continuous labeling during the efflux phase of the incubation. The synthesis rate of cholesterol in controls ranged from 20.7 to 38.8 nmol C_2-flux/mg/48 h (n = 8) with a mean value of 26.9 nmol C_2-flux/mg/48 h. Newly synthesized cholesteryl esters of controls varied between 5 and 10% of total cholesterol with a mean value of 6%. With both HDL$_3$ fractions, total cholesterol synthesis was induced and increased moderately, but dose dependently, from 28.8 ± 3.9 up to 39.7 ± 9.5 nmol C_2-flux/mg/48 h (fraction A) and from 30.5 ± 1.1 up to 36.7 ± 1.4 nmol C_2-flux/mg/48 h (fraction B) (Fig. 2).

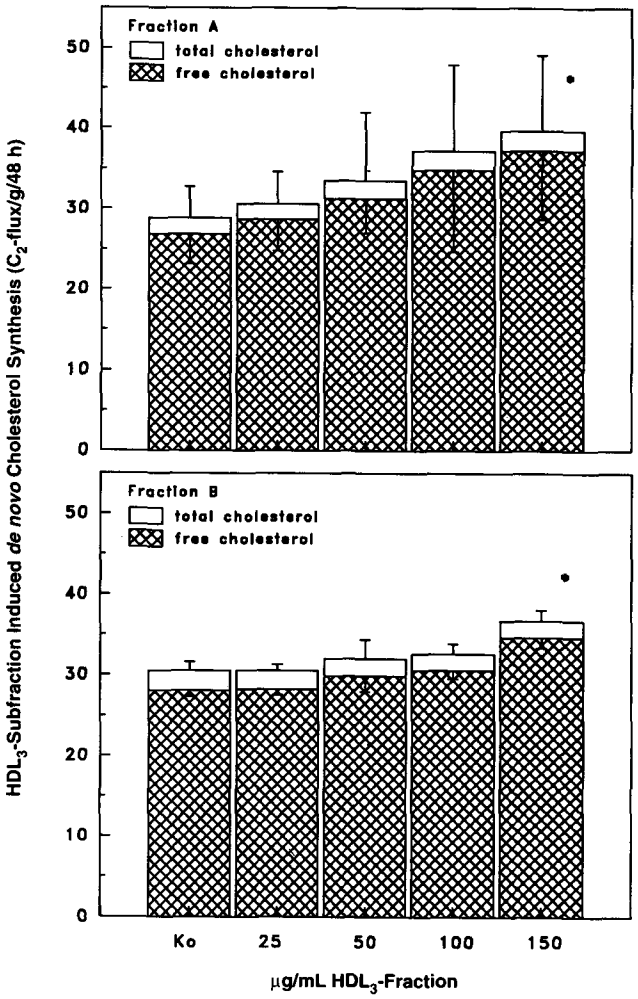

FIG. 2. HDL$_3$ subfraction induced changes in free, esterified and total cholesterol. Cells were treated as described in Table 1. Free and esterified cholesterol of cells and medium were extracted as described in the Materials and Methods section, and the values for cells and medium were combined. The data represent nmol C_2-flux/mg/48 h of three separate experiments ± 1 SEM *$P \leq 0.05$; abbreviations as in Figure 1.

Interesting changes in cholesterol distribution between cells and medium were also observed. The intracellular cholesterol moiety varied only minimally, but most of the HDL$_3$-induced, newly synthesized cholesterol was found in the culture medium. Compared to medium controls, the labeled free cholesterol in the medium was stimulated up to 173% (fraction A) and up to 143% (fraction B) (Table 1). This stimulation was found to be significant for both fractions at the highest concentration tested (Table 1), but remained just below the significance level, if both fractions were compared to each other.

Notably, both fractions predominantly effected an efflux of free as compared to esterified cholesterol into the medium. The C_2-flux into the ester portion in control cells did not exceed 8.7% of total cholesterol flux, and in the medium a maximal value of 6.8% of total was reached. Therefore, changes in esterified cholesterol affected only a very small amount as compared to free cholesterol. Nevertheless, a somewhat distinct distribution profile was observed for esters. With fraction A, total esterified cholesterol synthesis was slightly enhanced to 117 ± 20.5% of controls, whereas esterified cholesterol in

TABLE 1

HDL$_3$ Subfraction Mediated Changes of Labeled Free and Esterified Cholesterol[a]

HDL$_3$ (μg/mL)	Free cellular cholesterol	Free medium cholesterol	Esterified cell cholesterol	Esterified medium cholesterol
HDL$_3$ (fraction A)				
25	106.3 ± 4.1	109.7 ± 7.0	94.0 ± 12.1	105.3 ± 14.1
50	112.2 ± 3.1	123.5 ± 8.9	100.4 ± 3.0	126.8 ± 10.1
100	106.3 ± 7.4	155.6 ± 20.8	105.4 ± 4.0	132.1 ± 19.9
150	104.7 ± 8.3	173.3 ± 24.6[b]	95.6 ± 21.0	147.8 ± 31.2
HDL$_3$ (fraction B)				
25	103.1 ± 3.2	97.8 ± 5.4	88.3 ± 16.2	101.5 ± 4.0
50	103.4 ± 7.8	109.3 ± 8.8	88.0 ± 3.8[c]	89.4 ± 15.8
100	97.5 ± 2.8	120.8 ± 9.8	76.1 ± 6.9[c]	98.9 ± 25.7
150	104.7 ± 2.6	143.3 ± 13.1[c]	67.4 ± 13.1[b]	118.2 ± 24.7

[a]The data show changes in percentage of their corresponding control values measured separately in cells and in medium. Cells were grown in Dulbecco's minimal essential medium with 20% fetal calf serum (FCS) until day seven. Between days seven and nine, FCS was replaced by 10% lipoprotein deficient-FCS and only [^{14}C]octanoate was present for these 48 h. High density lipoprotein (HDL)$_3$ subfractions were added only for the last 24 h until day nine, without medium change. Free and esterified cholesterol of cells and medium were extracted separately as described in the Materials and Methods section. The C_2-fluxes (nmol/mg/48 h) are given as percent of controls ± SEM. For free cell cholesterol, the flux into controls was 12.79 ± 1.55 (fraction A) and 14.66 ± 0.85 (fraction B). For medium cholesterol the controls were 12.90 ± 2.45 (fraction A) and 13.46 ± 0.13 (fraction B). For esterified cell cholesterol, the controls refer to 1.16 ± 0.04 (fraction A) and 1.39 ± 0.19 (fraction B). For esterified medium cholesterol, the controls amounted to 0.84 ± 0.19 (A) and 0.98 ± 0.13 (B). The data represent the mean of three separate experiments.
[b]$P \leq 0.01$.
[c]$P \leq 0.05$.

the medium rose not significantly, up to 148% of controls, and the intracellular portion remained essentially constant (Table 1). With fraction B, total esterified cholesterol fell to 88 ± 11.1% of control values, whereas the esters in the medium reached 118%, and the cellular ester portion significantly decreased to 67% of controls (Table 1).

Kinetics of the HDL subfraction A- and B-mediated efflux. In this set of experiments, MVL was used as a precursor as well as [^{14}C]MVL as label, to bypass the regulatory enzyme hydroxymethylglutaryl coenzyme A (HMG–CoA) reductase and to effectively load the cells with cholesterol. In a separate experiment it was shown that CaCo-2 cells normally contained about 38.5 ± 4.1 µg cholesterol/mg protein (30.3 ± 2.0 µg/mg of free and 8.2 ± 2.0 µg/mg of esterified cholesterol, n = 6). The cholesterol content could be effectively enhanced by the incubation with MVL up to values of 52.9 ± 12.9 µg/mg (36.9 ± 12.5 µg/mg of free and 16.0 ± 4.0 µg/mg of esterified cholesterol, n = 4). Compared to albumin, which was present in controls and acted as an unspecific cholesterol acceptor, fraction A actively enhanced the basal cholesterol efflux dose dependently ($P \leq 0.05$) (Fig. 3A). It showed similar kinetics in cholesterol loaded cells, but with higher efficiency (Fig. 3C). The inhibition of cholesterol esterification during the labeling incubation reduced the induced efflux of label to the level of nonloaded cells (Fig. 3E), but remained significantly ($P \leq 0.05$) above that of controls. In contrast, fraction B enhanced the efflux in nonloaded cells significantly only at the highest concentration tested ($P \leq 0.05$) (Fig. 3B). Its effect on loaded cells was more pronounced (Fig. 3D), but did not reach the efficiency of fraction A. Compared to fraction A, its efflux potential could be completely blocked by the simultaneous inhibition of the esterification of the nascent labeled cholesterol (Fig. 3F).

Binding of [^{125}I]labeled liposomes, reconstituted from DMPC and apo (Lip A-I and Lip A-II). According to a previously described procedure (17) to characterize binding and the fate of HDL_3 (fraction A), similar experiments were performed with the apo A-containing liposomes. Labeled liposomes, Lip A-I and Lip A-II, exhibited specific binding to CaCo-2 cells at 4°C with saturation kinetics (Figs. 4 and 5). Equilibrium of binding at 4°C was reached after nearly 2 h for both Lip A-I and Lip A-II (data not shown). Derived from Scatchard analysis of the mean values, the binding characteristics of the liposomal particles were calculated. The B_{max} of Lip A-I amounted to 7445 ng/mg cell protein and the binding affinity K_D was 99.9 µg/mL (r = 0.920) (Fig. 4). The corresponding values for Lip A-II showed a B_{max} of 5024 ng/mg cell protein and a K_D of 47.3 µg/mL (r = 0.972) (Fig. 5).

In a separate series of experiments, Lip A-I and Lip A-II were prebound to the cells at 4°C, and their fate was followed during subsequent warm-up at 37°C (Fig. 6). The amount of the specifically membrane bound Lip A decreased rapidly to approximately half of the initial value within 1 h for Lip A-I and within 2 h for Lip A-II. Simultaneously, the medium release of intact particles showed a complementary rise (Fig. 6).

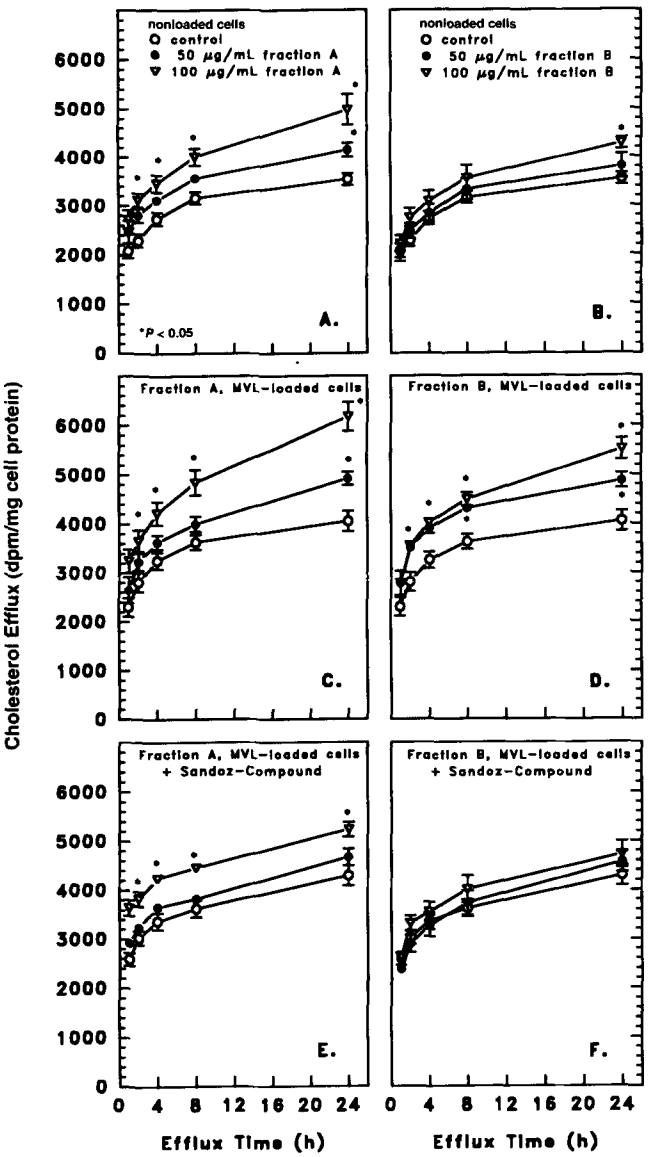

FIG. 3. Efflux kinetics mediated by HDL_3 subfractions A and B after cholesterol prelabeling. Cells were prepared as described in the Materials and Methods section. A + B demonstrate the efflux in nonloaded, prelabeled cells, C + D the efflux after preincubating of the cells with 5 mM mevalonolactone (MVL), E + F show changes after the additional supplementation with 5 µg/mL Sandoz-Compound 58035 during the labeling process to inhibit the esterification of newly formed cholesterol. The data represent the mean values of three to five separate experiments ± SEM. Significance levels are relative to controls. *$P \leq 0.05$; abbreviation as in Figure 1.

Studies with gold labeled Lip A-I and Lip A-II. The artificial liposomes reacted with gold similarly to the native HDL_3, as described previously (16,17). The labeled liposomes were identified by negative staining (Figs. 7 and 8). The particle volumes were comparable to native HDL_3 and resembled the native fraction A (data not shown). The membrane associated, labeled particles were predominantly found basolaterally. For both, internalization was shown by the membrane areas forming coated pits

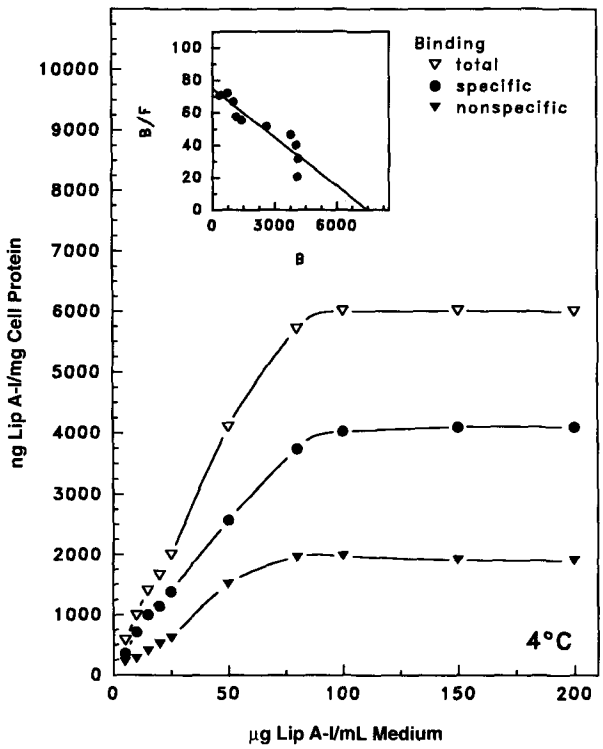

FIG. 4. Concentration dependent binding of liposome A-I (Lip A-I) at 4°C. Specific and unspecific binding were measured, and the Scatchard plot was calculated from the mean values. The data represent the mean values of two separate experiments (which varied by less than ±9%).

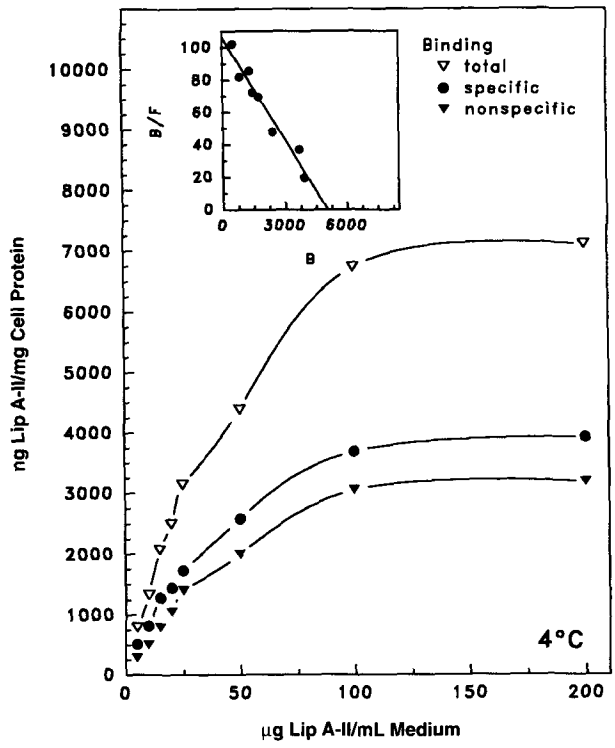

FIG. 5. Concentration dependent binding of liposome A-II (Lip A-II) at 4°C. Specific and unspecific binding were measured, and the Scatchard plot was calculated from the mean values. The data represent the mean values of two separate experiments (which varied by less than ±15%).

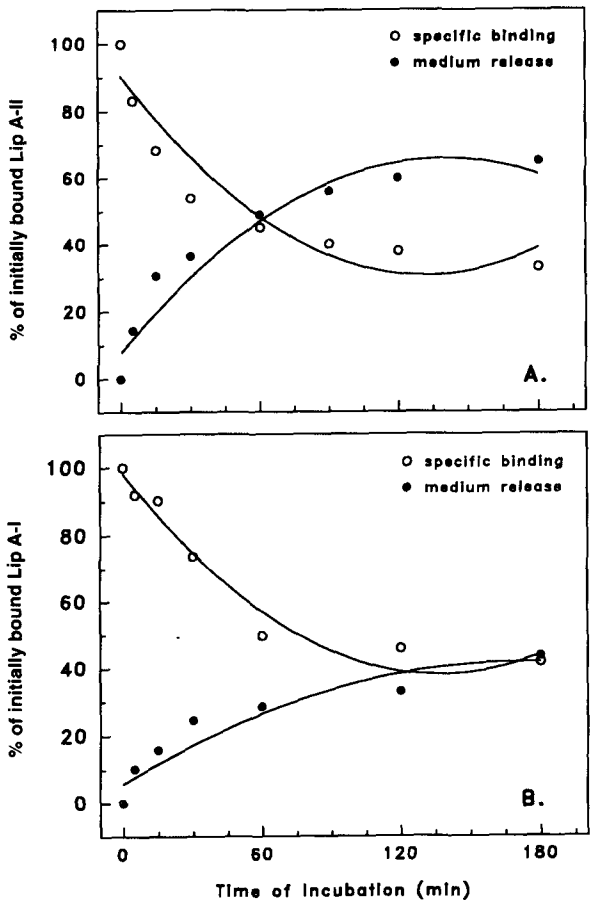

FIG. 6. Medium release of prebound liposome A-I (Lip A-I) and liposome A-II (Lip A-II). At day five, cells were incubated with 10 µg/mL [^{125}I]Lip A-I or [^{125}I]Lip A-II for 2 h at 4°C, to allow specific binding. Subsequent warm-up to 37°C after removing the medium and intensively washing the cells led to a time-dependent reduction of membrane bound [^{125}I]Lip A-I (A) and [^{125}I]Lip A-II (B) and a simultaneous appearance of intact labeled particles in the medium. The data represent the mean values of two separate experiments (which varied by less than ±13%).

as well as by internalized endosomal gold-labeled particles (Figs. 7 and 8). Lip A-I also was detected close to partially dissolved lipid droplets (Fig. 7D). Both liposomal particles were found in normal as well as in bloated endosomes, containing curled membrane fragments.

Efflux kinetics of Lip A-I-mediated efflux after cholesterol prelabeling. Lip A-I were used to monitor the efflux capacity of liposomal particles. Similar to the native lipoprotein fractions, Lip A-I was added after prelabeling the cells as described above. Interestingly, both concentrations of the liposomal fraction used resulted in a similar efflux efficiency (Fig. 9), which was slightly higher than that observed with fraction A. This was true for non- and MVL-loaded cells (Fig. 9A,B). The cells, additionally incubated with Sandoz-Compound 58035, also showed a very high efflux (Fig. 9C), much higher than that observed with fraction A.

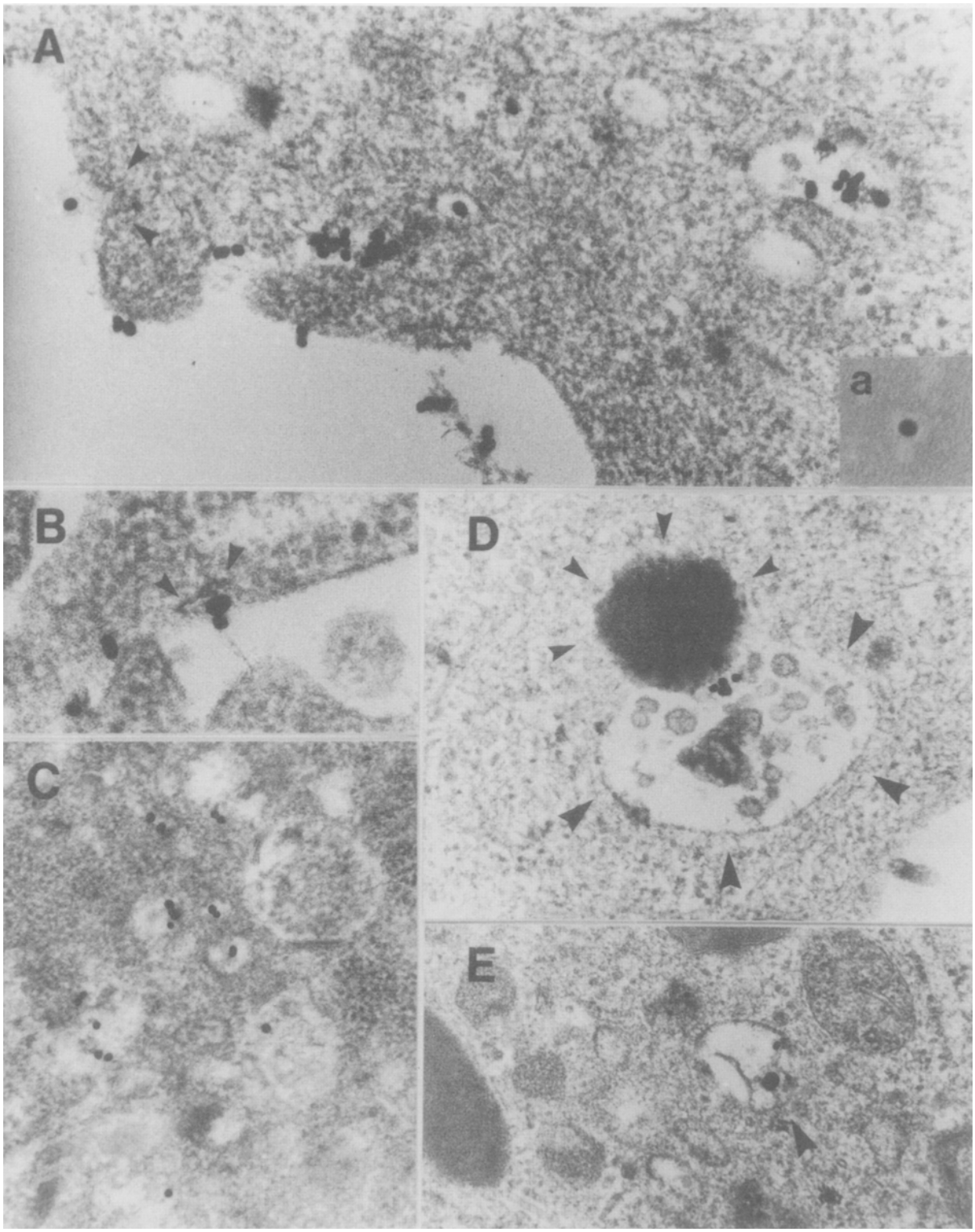

FIG. 7. Electron microscopy of gold labeled liposome A-I (Lip A-I). The fate of gold-labeled liposomes was followed after a 2-h incubation at 37°C, washing the cells and subsequent fixation. Basolateral binding as well as internalization was seen. (A) Electron micrograph shows binding, formation of a coated pit (small arrow heads), and endosomal uptake and intracellular transport (arrows) of the gold-labeled liposomes; magnification (M), 80,000-fold. The right lower corner shows the gold-liposome complex by negative staining (a). (B) Membrane binding site, forming a coated pit, M, 110,000-fold. (C) Multiple endosomal structures, containing the gold label; M, 60,000-fold. (D) Bloated endosome (small arrow heads) in direct contact with the remainder of a lipid droplet (small arrows). The endosome contains curled membrane fragments; M, 60,000-fold. (E) Another gold label in an endosomal structure, also containing residual membrane fragments; M, 60,000-fold.

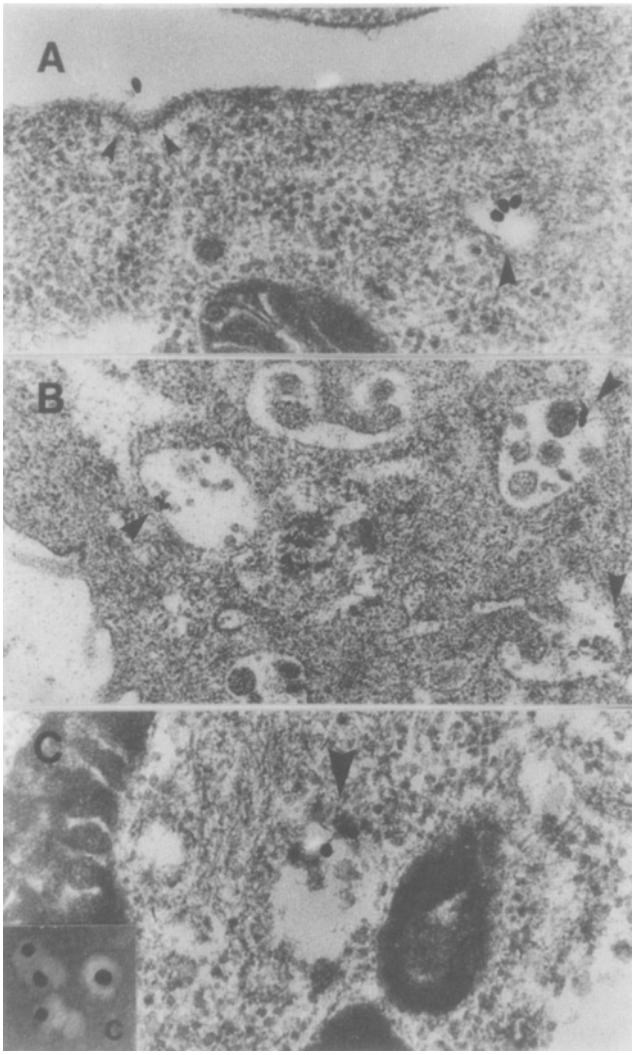

FIG. 8. Electron microscopy of gold-labeled liposome A-II (Lip A-II). The fate of gold-labeled liposomes was followed after a 2-h incubation at 37°C, washing the cells, and subsequent fixation. Basolateral binding as well as internalization was seen. (A) Membrane associated gold label, inducing the formation of a coated pit (small arrow heads) and gold-labeled Lip A-II located in an endosome; magnification (M) 80,000-fold. (B) Some bloated endosomal structures, containing membrane fragments, as shown for Lip A-I; M 40,000-fold. (C) Demonstration of such an endosomal structure in greater detail; M 80,000-fold. Left lower corner, negative staining of the gold-liposome complex (c).

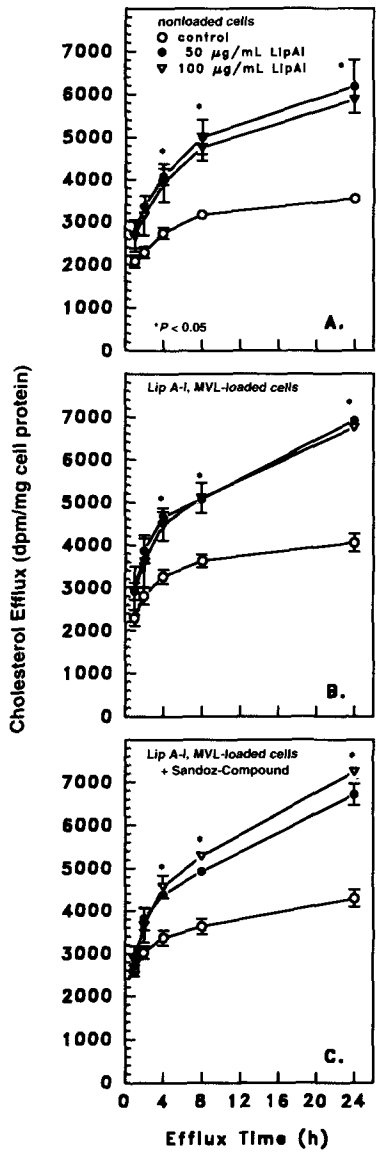

FIG. 9. Efflux kinetics of liposome A- (Lip A-I) after cholesterol prelabeling. Cells were prepared and treated as described in the Materials and Methods section. The experimental design corresponded to that in Figure 3. (A) shows the efflux in nonloaded cells; (B) the efflux after preloading of the cells with 5 mM MVL; (C) shows changes after additional supplementation with 5 µg/mL Sandoz-Compound 58035 during the labeling process to inhibit the esterification of newly formed cholesterol. The data represent the mean values of three separate experiments ±1 SEM. Significance levels refer to controls. *$P \leq 0.05$. See Figure 3 for abbreviation.

DISCUSSION

In cultured enterocytes, HDL-mediated efflux was recently shown to involve a complicated pathway of cell surface binding, endosomal internalization and lysis of lipid droplets, followed by retroendocytosis of the particles (16,17). Thus, unlike HDL_2, which delivers cholesterol to cells in a similar manner to LDL (25,27,28), HDL_3 has its own receptor pathway (11–17) and acts as a cholesterol acceptor. During the previous studies, the HDL_3 subfraction used was equivalent to fraction A in the present study, i.e., it contained only apo A-I. However, because HDL_3 prepared by ultracentrifugation contains other apo, in particular A-II, it was of interest to compare fractions differing in their apo composition. The apo and lipid compositions clearly depend on the method of isolation and have been shown to affect binding and probably cholesterol efflux (33–36,38,39). However, studies in macrophages with particles differing in the apo A-I/apo A-II ratio obtained by preparative isoelectric focusing (36) or isotachyphoresis (35) gave opposite results. When different particle populations containing either apo A-I or apo A-I plus apo A-II were ob-

tained by immunoaffinity chromatography (34), only the apo A-I containing fraction promoted cholesterol efflux. Nevertheless, both particles displayed specific binding within the same range of concentrations. Another approach used the substitution of apo A-I by apo A-II in intact HDL_3 (38) or a combination of immunological methods and apo A-II enrichment of HDL_3 (39). These well designed binding experiments led us to postulate that the mechanisms involved probably differ between apo A-I and apo A-II containing particles. The lack of agreement in the respective literature is probably due to differences in particle preparation resulting in variable particle compositions. Therefore, it seemed appropriate to use native particles of different apo compositions obtained by a method which was able to clearly separate two quantitatively important subfractions, a pure apo A-I fraction from the mixed fraction. The characterization of the binding properties of the two fractions described above revealed a higher specific binding affinity of the apo A-I particles (fraction A) as compared to the apo A-I/apo A-II containing fraction B, but the maximal binding capacity of both fractions was similar. An inverse affinity pattern between distinct apo A-I and apo A-I/apo A-II containing particles was reported for endothelial cells (39). No differences, depending on the apo composition, were observed in adipose cells (34) and macrophages (36). Thus, intestinal epithelial cells appear to differ with respect to these binding patterns from most other cell types.

Considering the differences in binding of both fractions, it was of interest to study the efflux characteristics. In one experiment, cholesterol synthesis was labeled before (24 h) and during (24 h) the incubation with the HDL_3 subfractions. With the use of the label [^{14}C]octanoate, the mechanisms of feedback regulation remained intact and accumulation of excess cholesterol could be avoided. The amount of labeled cellular cholesteryl esters did not exceed 10% of the total cholesterol label. Both fractions mainly stimulated the synthesis of free cholesterol dose dependently in these nonloaded cells, whereas the ester portion remained nearly unchanged. Fraction A was more effective in releasing cholesterol particularly at lower concentrations. Interestingly, the excess cholesterol was completely found in the medium, whereas the portion of cellular free cholesterol remained unchanged. Changes of the very small amount of esterified cholesterol were quantitatively less important, although a significant reduction in the amount of labeled cellular esters by fraction B was noted. The results indicated a net loss of free cholesterol, which was apparently balanced by the induction of synthesis. The findings were compatible with a HMG–CoA reductase (EC 1.1.1.34) induction by unfractionated HDL_3 observed in rat intestinal crypt cells (27) and CaCo-2 cells (52). Simultaneously, an inhibition of acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) was observed, which explains decreased cholesteryl ester formation (52).

At any rate, simple cholesterol exchange mechanisms (53) simulating the observed net efflux of labeled cholesterol may be excluded. A reduction of cellular cholesterol mass (free and esterified) was observed previously in cholesterol and cholesteryl ester loaded cells (17) and recently also in normal cells (unpublished data). Loading with MVL effectively enhanced total cellular cholesterol, mainly in the esterified form, and enlarged the efflux sensitive cholesterol pool. As recently shown, cholesterol enriched cells displayed more HDL receptors because they are subject to up- and downregulation, depending on the cellular cholesterol content (6,17). This may explain why the amount of cholesterol efflux, particularly by fraction A, was related to the intracellular cholesterol excess.

The medium cholesterol detected in control experiments may be due to serum proteins, especially albumin acting as potential acceptors (54, and unpublished data). In addition, CaCo-2 cells synthesize and secrete lipoproteins basolaterally as has been shown previously (43,55). Compared to the secretion of triglycerides and phospholipids, the amount of secreted cholesteryl esters was low, especially when the medium contained LPD-FCS (43). Because the ester portion in our medium was also very low, it seems possible that small amounts of cholesteryl esters were actively secreted by lipoprotein formation. However, such a contribution is unlikely to be specifically induced by HDL_3 and therefore does not explain the differential between controls and under HDL_3 supplemented conditions.

To further discriminate the observed relationship in cholesterol synthesis and efflux, efflux was studied in normal and cholesterol-enriched cells which had been labeled with [^{14}C]MVL. Although the kinetics of efflux in the presence of HDL_3 were quite similar in nonloaded and loaded cells, the efflux efficiency in loaded cells was higher for both fractions. Compared to fraction A, fraction B was less effective in promoting efflux from nonloaded cells which were low in cholesteryl esters. Fraction A also mediated efflux of labeled cholesterol in mevalonolactone loaded cells if the esterification of the labeled, newly synthesized cholesterol was blocked by an ACAT-inhibitor, whereas fraction B did not. The total amount of fraction A-induced efflux was reduced as compared to loaded but untreated cells, suggesting that accessible pools of cholesteryl esters were reduced. These findings provide evidence that the apo A-II containing fraction B predominantly mobilized esterified cholesterol and was less effective, compared to fraction A, in mediating efflux of excess free cholesterol. Electron microscopic observations demonstrated a direct interaction between endosomes, containing labeled fraction A (16,17) or labeled liposomes and lipid droplets. It may be hypothesized that these cholesteryl ester stores and their hydrolysis represent the cholesterol substrate for efflux. Recent work had also demonstrated intracellular traffic of HDL (56), involvement of the trans-Golgi system (56,57), and possibly exocytosis as HDL_2-like, apo E enriched particles (58).

Previous results reported for other cells in regard to cholesterol efflux mediated by lipoprotein particles either containing apo A-I or apo A-I and apo A-II have been controversial. With native lipoprotein fractions of varying apo A-I/apo A-II ratios, a predominant role of

apo A-I had been demonstrated in adipose cells, whereas efflux was inhibited with increasing apo A-II concentrations (34). Another study on the same cell line showed that cholesterol efflux was promoted only by native HDL_3 particles containing apo A-I (33). Artificial liposomes of different apo A-I/apo A-II ratios also had an efflux potential which decreased with an increase in apo A-II content (33). The preparation of six distinct subpopulations of HDL, differing in their apo A-I/apo A-II ratios, had comparable effects on the efflux from peritoneal macrophages (36), while other macrophage studies (35) identified apo A-I as the predominant apo. Johnson *et al.* (37) found either normal or cholesterol loaded, no particle specific differences in three cell types. From these data it could be concluded that apo A-I plays the dominant role in mediating efflux (4, 33–35), whereas apo A-II may even be antagonistic to the process (33,34). The present results in enterocytes are consistent with a reduced efflux potential of apo A-II, but for the first time suggest cholesteryl ester efflux by apo A-II containing particles.

For further characterization, the binding of apo A-I and of apo A-II was studied separately, using previously described liposomal preparations (33,59). These liposomes mainly differed in their lipid composition from native or apo A-II enriched HDL_3 and less so in size. Compared to the apo A-I subfraction A, the apo A containing liposomes differed with respect to binding affinity and maximal binding capacity. Lip A-I showed a higher B_{max} and lower affinity than the corresponding native particle, whereas Lip A-II resembled fraction A in affinity. Similar but lower binding affinities were found with apo A-I and apo A-II liposomes in rat ovaries (60). It remains to be clarified why the measured K_D values of native and reconstituted particles were somewhat higher as compared to other experimental systems (34–36) dealing with different particles derived from plasma. Both gold-labeled liposome particles displayed endocytosis, intracellular endosomal packing and contacts to lipid droplets, as shown for HDL_3 (17). Prebound Lip A-I and Lip A-II were released as intact particles, similar to the native particles (17). Taken together, these findings also support the retroendocytosis concept for liposomal particles.

Finally, the liposomal binding and uptake studies were complemented by experiments demonstrating that Lip A-I induced efflux. Lip A-I induced an efflux of cholesterol at a maximal rate, which was slightly above that of fraction A. Unlike fractions A and B, efflux was not reduced if esterification was inhibited, suggesting that accessible pools of free cholesterol were depleted.

In conclusion, efflux efficiency was influenced by the cholesterol and ester content of the cells and by the apo pattern of the HDL_3 particles. There is evidence that both apo, A-I and A-II, were bound specifically and were retroendocytosed. Apo A-I played the dominant role for the receptor mediated cholesterol efflux in cultured enterocytes, but the apo A-II content did not prevent efflux. The apo A-II content may preferentially account for the mobilization of cellular cholesteryl esters, therefore mainly promoting efflux from cholesterol-enriched cells.

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The Effects of Clofibrate and Bezafibrate on Cholesterol Metabolism in the Liver of the Male Rat

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Fibric acid derivatives are used to treat hyperlipidemia and have wide ranging effects on lipid metabolism. The action of these compounds on cholesterol esterification, catalyzed by acyl-coenzyme A:cholesterol acyltransferase (ACAT), has been quite widely studied, but their effect on cholesteryl ester hydrolysis and the enzyme neutral cholesteryl ester hydrolase (nCEH) has been largely ignored. Male rats were therefore fed for 10 d on a standard chow diet supplemented with either clofibrate or bezafibrate, to study their effects on plasma lipid levels and hepatic cholesterol metabolism. Plasma triacylglycerols were not significantly altered by these diets, but bezafibrate significantly lowered plasma cholesterol levels (29.7%, $P < 0.01$). When expressed per unit weight of DNA, both fibrates reduced the hepatic content of triacylglycerol, cholesterol and cholesteryl esters (40, 18.7, 16.5 and 66.7, 28.6, 34.2% for clofibrate and bezafibrate, respectively). ACAT activity was significantly reduced by both drugs, but clofibrate (65% inhibition) was more effective than bezafibrate (35% inhibition). The most dramatic effect of the diets was a marked increase in the activity of both the microsomal and the cytosolic nCEH. When expressed on a whole liver basis, the effect of bezafibrate on the cytosolic enzyme (13.6-fold increase in activity) was much greater than that of clofibrate (4.8-fold increase). Increases in the activity of a cytosolic protein that inhibits the activity of nCEH were also noted, but these changes were relatively small. The results suggest that the activation of nCEH, in combination with the inhibition in ACAT activity, contributes to a decrease in the cholesteryl ester content of the liver which may influence the secretion of very low density lipoprotein.
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The fibric acid derivatives clofibrate and bezafibrate are used in the treatment of hyperlipidemia in patients at risk from coronary artery disease and in some diabetics whose hyperlipidemia cannot be controlled by other means. Despite their wide use, the mechanisms by which these compounds exert their hypolipidemic effects are not yet clear but seem to involve both increased clearance of very low density lipoprotein (VLDL), through activation of lipoprotein lipase, and, to a lesser extent, to a decreased production of triglycerides (1–3). In addition, these agents cause substantial reductions in plasma cholesterol (4–6) although again the mechanism of this effect is unknown.

The hepatic metabolism of cholesterol involves four key enzymes as well as receptors, controlling the uptake of lipoprotein particles from plasma. The synthesis of cholesterol is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase while cholesterol 7α-hydroxylase governs the catabolism of cholesterol to bile acids. The formation of cholesteryl esters, the storage form of cholesterol in the liver, is catalyzed by acyl-coenzyme A:cholesterol acyltransferase (ACAT) (7,8) while the hydrolysis of the esters back to free cholesterol is due to the action of a neutral cholesteryl ester hydrolase (nCEH) (9). Most of the fibric acid derivatives appear to inhibit ACAT (4,5,9) while the more potent derivatives, such as bezafibrate, also inhibit HMG-CoA reductase and cholesterol 7α-hydroxylase (4,5,10) and, at the same time, stimulate cholesterol excretion by increasing the biliary output of the sterol (11,12).

An important aspect of hepatic cholesterol metabolism that appears to have been largely neglected is the possible involvement of nCEH in the mechanism of action of these hypolipidemic agents. It may, nevertheless, have a major role since its substrate, the pool of cholesteryl esters, will be influenced by the decline in ACAT activity. Such an effect has been suggested to explain the fibrate-induced loss of cholesteryl esters from cultured 3T3 fibroblasts (13,14).

In addition to possible variations in the activity of the nCEH, we have also shown that the activity of a hepatic protein inhibitor of this enzyme can be varied under a variety of hormonal and nutritional conditions (15,16). It is possible, therefore, that these hypolipidemic agents would also affect the activity of this protein, so altering nCEH activity.

In the present study we have fed clofibrate and bezafibrate to male rats and have demonstrated that, in addition to inhibiting ACAT and altering the cholesterol content of the liver, a major effect of these drugs was to cause a marked increase in the activity of hepatic nCEH, both cytosolic and microsomal. Minor changes in the activity of the cytosolic inhibitor of nCEH were also noted.

MATERIALS AND METHODS

[9,10(n)-^{3}H]Oleic acid (370 GBq/mmol), [9,10(n)-^{3}H]triolein (370 GBq/mmol), [1-^{14}C]oleic acid (2 GBq/mmol) and cholesteryl [1-^{14}C]oleate (2 GBq/mmol) were obtained from Amersham (Little Chalfont, United Kingdom). Cholesteryl [9,10(n)-^{3}H]oleate and [9,10(n)-^{3}H]oleoyl CoA were synthesized as described (15,17). 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 g at the start of the experiment, were kept on a constant light/dark cycle (lights on 08.00–20.00 h) and were fed for 10 d on 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. One group of rats was fed the control diet while two other groups were fed the same basic diet supplemented with either clofibrate or bezafibrate (0.3% w/w). These

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Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; C, cholesterol; CE, cholesteryl ester; nCEH, neutral cholesteryl ester hydrolase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SEM, standard error of the mean; TRIS, tris(hydroxymethyl)-aminomethane; VLDL, very low density lipoprotein.

agents were incorporated by mixing an acetone solution with the crushed diet and then allowing the solvent to evaporate for at least 48 h. The control diet was treated in the same way but with acetone alone. The rats were fed at 08.30 h at a 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 09.30 h, 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 plasma samples stored at -20°C.

Tissue processing. Livers were homogenized as described (17) 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.* (18) 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 (20 nmol, 250,000 dpm) to cholesteryl [9,10(n)-^{3}H]oleate as previously described using endogenous cholesterol as the substrate in a total volume of 0.2 mL (17). 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 (19) using cholesterol and phosphatidylcholine in a molar ratio of 1:2. All assays were preincubated for 30 min before addition of 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 nCEH was measured, essentially according to Shand and West (15) using 50 μg of either microsomal or cytosolic protein in a total volume of 0.2 mL. The reaction was initiated by 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.

Triacylglycerol lipase in cytosols was assayed in an identical manner to nCEH using [9,10(n)-^{3}H]triolein as substrate.

Assay of the nCEH inhibitor protein. The liver cytosols were assayed for inhibitory activity against rat mammary gland microsomal nCEH essentially as described (15) using 30–40 μg of cytosolic protein. The quantity of cytosolic protein required to produce 50% inhibition of the nCEH was then calculated. Controls, without mammary gland microsomes, were incubated at the same time to allow correction for the activity of cytosolic nCEH.

Estimation of cholesterol and cholesteryl esters. Lipids, extracted (20) 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. The extracts were then assayed, with and without cholesteryl esterase, according to the fluorimetric method of Gamble *et al.* (21), to give the microsomal contents of total and free cholesterol, respectively. Esterified cholesterol could then be estimated by difference.

Estimation of triacylglycerols. Triacylglycerols 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.* (22) 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 (23), using bovine serum albumin as standard, and DNA as described by Labarca and Paigen (24).

RESULTS

Body weight, liver weight and hepatic and plasma lipid levels. Neither of the dietary supplements affected the rate of body weight gain in the course of the experiment, but, in agreement with previous reports, liver weight was significantly increased by both clofibrate (21%; $P < 0.05$) and bezafibrate (50%; $P < 0.01$) by the end of the experiment (Table 1). The concentration of cholesterol in the plasma tended to be lower in the animals treated with the fibrates (Table 1), but only with bezafibrate did this achieve significance ($P < 0.05$). Both drugs also tended to decrease plasma triacylglycerol levels but neither produced a significant effect. The triacylglycerol levels in the liver, whether expressed on a cellular (i.e., related to DNA concentration) or whole liver basis, were significantly lowered by the dietary treatments (Table 2). Bezafibrate was more effective than was clofibrate since it reduced cellular and whole liver triacylglycerol by 63% ($P < 0.001$) and 32% ($P < 0.01$), respectively, compared with 38% ($P < 0.001$) and 15% ($P < 0.05$) for clofibrate.

The dietary supplementation with the fibric acid derivatives caused significant reductions in the levels of both free and esterified cholesterol in the liver when expressed on a DNA basis (Table 2), the most effective again being bezafibrate. However, when expressed on a whole liver basis, the differences disappeared.

TABLE 1

The Effect of Treatment with Clofibrate and Bezafibrate on Liver Weight and Plasma Lipid Levels[a]

Treatment	Liver weight (g)	Plasma	
		Triacylglycerol (mg/100 mL)	Total cholesterol (mg/100 mL)
Control (11)	8.4 ± 0.4	50.0 ± 2.6	44.1 ± 2.9
Clofibrate (6)	10.2 ± 0.5[b]	45.9 ± 2.5	40.0 ± 5.2
Bezafibrate (5)	12.6 ± 1.0[c]	45.5 ± 3.3	31.0 ± 2.1[c]

[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 were determined using the Student's *t*-test.
[b]$P < 0.05$.
[c]$P < 0.01$.

TABLE 2

The Effect of Treatment with Clofibrate and Bezafibrate on Liver Lipid Levels[a]

Treatment	Triacylglycerol		Cholesterol		Cholesteryl esters	
	mg/mg DNA	mg/total liver	μg/mg DNA	mg/total liver	μg/mg DNA	mg/total liver
Control (11)	1.5 ± 0.1	77.8 ± 2.4	261.4 ± 15.0	15.5 ± 0.7	168.0 ± 7.6	10.7 ± 0.6
Clofibrate (6)	0.9 ± 0.06[d]	66.5 ± 4.4[b]	212.5 ± 3.9[c]	15.8 ± 0.3	140.2 ± 8.9[b]	10.4 ± 0.7
Bezafibrate (5)	0.5 ± 0.06[d]	53.2 ± 4.4[c]	186.6 ± 6.8[d]	19.1 ± 1.9	110.6 ± 4.8[d]	11.1 ± 0.7

[a]Liver triacylglycerol levels were determined directly in the tissue homogenates whereas liver cholesterol and cholesteryl esters were measured by means of a fluorimetric 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 were determined using the Student's *t*-test. [b]$P < 0.05$. [c]$P < 0.01$. [d]$P < 0.001$.

ACAT and microsomal cholesterol and cholesteryl esters. The effects of dietary fibric acid derivatives on ACAT activity are shown in Table 3. When assayed using the cholesterol endogenous to the microsomes, clofibrate reduced the activity of ACAT by about 65% ($P < 0.001$) and in this instance was more effective than bezafibrate which caused only a 35% ($P < 0.01$) reduction in the activity of the enzyme. When exogenous cholesterol was included in the assay, the difference between the controls and the clofibrate-treated animals was maintained whereas the effect of bezafibrate became insignificant. The total ACAT activity in the whole liver was not significantly changed by bezafibrate but was reduced by about 56% ($P < 0.001$) by clofibrate.

A similar result was observed in relation to the levels of microsomal free and esterified cholesterol (Table 4) where clofibrate reduced these values to a greater extent than did bezafibrate. The ratio of free to esterified cholesterol in the microsomes was not affected by clofibrate treatment but was significantly raised by bezafibrate.

TABLE 3

The Effect of Treatment with Clofibrate and Bezafibrate on ACAT Activity[a]

Treatment	ACAT activity		
	pmol CE formed per min/mg microsomal protein		nmol CE formed per min/total liver
	Endogenous cholesterol	Exogenous cholesterol	
Control (11)	231.6 ± 19.5	597.9 ± 65.9	23.0 ± 1.8
Clofibrate (6)	79.9 ± 12.3[c]	226.8 ± 34.9[c]	10.1 ± 1.4[c]
Bezafibrate (5)	151.2 ± 14.1[b]	435.6 ± 52.1	20.0 ± 2.9

[a]Acyl-CoA:cholesterol acyltransferase (ACAT) activities were assayed using 150 μg microsomal protein incubated with [9,10-^{3}H]oleoylCoA 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. The "total liver" figures are based on the assay using endogenous cholesterol. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values were determined using the Student's *t*-test. CE, cholesteryl ester.
[b]$P < 0.01$.
[c]$P < 0.001$.

The changes in ACAT activity showed a positive correlation with the levels of both free (r = 0.60) and esterified cholesterol (r = 0.54) in the microsomal membranes (Fig. 1).

Microsomal and cytosolic cholesteryl ester hydrolase (nCEH). The most dramatic effects produced by the fibrate-supplemented diets were changes in the activities of both microsomal and cytosolic nCEH (Table 5). On a protein basis, clofibrate led to a 4.5-fold increase in the cytosolic nCEH while bezafibrate increased the enzyme activity by a factor of 8.6. When the increase in liver weight was taken into account, it was apparent that the increases in cytosolic nCEH activity were even greater. The microsomal nCEH was also increased by the fibric acid derivatives although the differences were not quite as marked as seen with the cytosolic enzyme with both drugs causing a 3- to 4-fold increase in enzyme activity. There was a significant, negative correlation (r = 0.91) between the microsomal nCEH activities and the levels of microsomal cholesteryl esters (Fig. 2). In contrast, the levels of cytosolic triacylglycerol lipase were unaffected by any of these diets (data not shown).

Cytosolic inhibitory protein. Table 6 shows the effects of the dietary treatments on the activity of the liver cytosolic inhibitor of nCEH. Whereas clofibrate caused no significant changes in the activity of the inhibitor when expressed on a protein basis, bezafibrate treatment led to a decrease in inhibitory activity of 22% ($P < 0.01$). When ex-

TABLE 4

The Effect of Treatment with Clofibrate and Bezafibrate on the Microsomal Contents of Cholesterol and Cholesteryl Esters[a]

Treatment	Cholesterol (μg/mg protein)	Cholesteryl esters (μg/mg protein)	C/CE ratio
Control (11)	31.4 ± 1.3	19.0 ± 1.0	1.63 ± 0.05
Clofibrate (6)	19.3 ± 0.9[d]	11.3 ± 0.8[d]	1.73 ± 0.07
Bezafibrate (5)	26.0 ± 0.8[c]	13.2 ± 0.7[d]	2.00 ± 0.15[b]

[a]Cholesterol (C) and cholesteryl ester (CE) concentrations were measured by means of a fluorimetric assay on ethanolic solutions of the extracted microsomal lipids. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values were determined using the Student's *t*-test.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

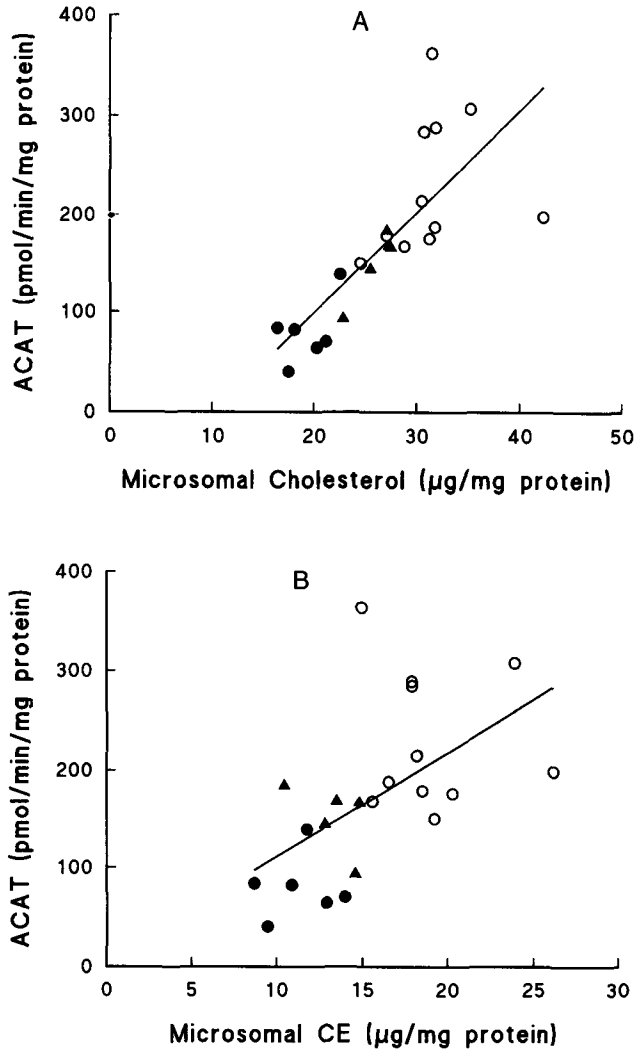

FIG. 1. Scatter diagram relating acyl-CoA:cholesterol acyltransferase (ACAT) activities to the microsomal contents of cholesterol (A) and cholesteryl esters (CE) (B) in the livers of control rats (○) and animals treated with clofibrate (●) and bezafibrate (▲). Regression analysis was performed and showed the relationships to be linear for both ACAT vs. cholesterol (y = 10.28x - 105.99, r = 0.77) and ACAT vs. CE (y = 10.64x + 4.75, r = 0.56).

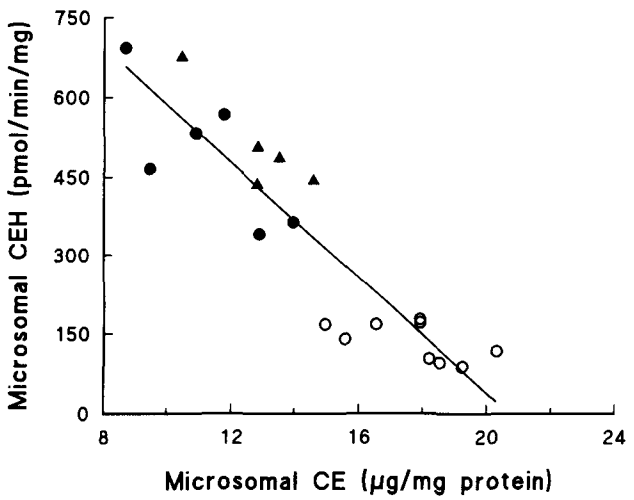

FIG. 2. Scatter diagram relating microsomal neutral cholesteryl ester hydrolase (nCEH) activities to the microsomal contents of cholesteryl esters (CE) in the livers of control rats (○) and animals treated with clofibrate (●) and bezafibrate (▲). Regression analysis was performed and showed the relationship to be linear (y = -54.71x + 1133.34, r = -0.91).

pressed on a whole liver basis, however, it was apparent that both fibrates had increased the total amount of inhibitor present in the tissue by around 21% ($P < 0.05$). In general, however, there appeared to be little or no correlation between the activities of the cytosolic inhibitor and those of the microsomal or cytosolic nCEH enzymes.

DISCUSSION

The inhibition of ACAT activity recorded in the present study confirms the observations of other workers (4,9). The level of ACAT activity is normally dependent on the availability of cholesterol substrate, and there was a good correlation between ACAT activity and microsomal cholesterol content suggesting that a decrease in the availability of substrate contributed to the effect. However, ACAT activity in isolated microsomes can also be increased by the provision of exogenous cholesterol, and with such treatment the ACAT activity in the microsomes

TABLE 5

The Effects of Treatment with Clofibrate and Bezafibrate on the Activities of the Microsomal and Cytosolic Cholesteryl Ester Hydrolases (CEH)[a]

	Microsomal CEH		Cytosolic CEH	
Treatment	pmol/min/mg protein	nmol/min/liver	pmol/min/mg protein	nmol/min/liver
Control (11)	139.8 ± 10.3	13.9 ± 0.8	11.7 ± 1.5	12.2 ± 1.6
Clofibrate (6)	494.2 ± 49.3[b]	63.3 ± 5.8[b]	52.6 ± 7.8[b]	68.8 ± 8.7[b]
Bezafibrate (5)	509.8 ± 38.9[b]	66.9 ± 7.0[b]	100.9 ± 11.3[b]	166.7 ± 25.5[b]

[a]CEH activities were assayed on 50 μg of microsomal or cytosolic protein incubated with cholesteryl [9,10-^{3}H]oleate for 45 min. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values were determined using the Student's *t*-test.
[b]$P < 0.001$.

TABLE 6

The Effects of Treatment with Clofibrate and Bezafibrate on the Activity of the Cytosolic Protein Inhibitor of Cholesteryl Ester Hydrolase (CEH)[a]

Treatment	CEH inhibitor activity	
	% Inhibition by 40 μg cytosolic protein	50% Inhibitory units/liver ($\times 10^3$)
Control (9)	37.0 ± 1.5	19.0 ± 0.9
Clofibrate (5)	35.3 ± 1.5	23.8 ± 1.5[b]
Bezafibrate (5)	28.8 ± 2.2[c]	23.0 ± 1.1[b]

[a]The activity of the neutral CEH inhibitor protein was assayed in the liver cytosols by incubation of 30–40 μg of cytosolic protein with rat mammary gland microsomes. The percentage inhibition of the CEH activity due to 40 μg was then determined. The activity in whole liver is expressed as 50% inhibitory units where 1 unit is the amount (μg) of cytosolic protein required to inhibit the mammary microsomal CEH by 50%. The results are the means ± SEM for the number of animals given in parentheses. Significant differences from control values were determined using the Student's *t*-test.
[b]$P < 0.05$.
[c]$P < 0.01$.

from the bezafibrate-treated animals was seen to be similar to that of the controls. The remaining small difference between the two groups of animals could be accounted for by the increased nCEH activity in the bezafibrate-treated microsomes, even though nCEH activity recorded under the conditions of the ACAT assay was reduced by 50% in the absence of exogenous cholesterol, and by 80% when cholesterol was added. This suggests that the levels of ACAT enzyme in the two sets of animals are the same and that the apparent differences were a reflection of substrate availability in the microsomes, a conclusion supported by the total liver ACAT measurements (Table 3). However it was apparent that, for the clofibrate-treated animals, ACAT activity in the presence of exogenous cholesterol was still considerably less than that seen in the controls. This indicates that in those animals the reduction in ACAT activity arose from a decrease in the amount of the enzyme as well as a decrease in the availability of cholesterol. Again this conclusion is supported by the results obtained when ACAT activity was calculated for the whole liver (Table 3).

A reduced level of cholesteryl esters in the livers of clofibrate-treated rats has been reported by Turley and Dietschy (11) while Stahlberg *et al.* (4,5) observed a lowered hepatic microsomal content of esterified cholesterol. Reduced accumulation of cholesteryl esters was also recorded by Hudson and Day (13) when 3T3 fibroblasts were incubated with fibric acid derivatives. Although this could have been an effect of the reduction in ACAT activity, they also found that the large accumulation of cholesteryl esters, induced in fibroblasts by incubation with modified low density lipoprotein, was rapidly lost when either clofibrate or bezafibrate was added to the medium. They concluded that this could not arise solely from inhibition of ACAT and, since esterified cholesterol must be hydrolyzed prior to removal from the cell, speculated that increased hydrolysis of the esters may have been involved (13).

Although the fibrates were shown to have no direct effect on the lysosomal CEH (14), the results presented in the current paper confirm this hypothesis by demonstrating that nCEH in both the microsomal and cytosolic fractions was markedly increased in the livers of rats treated with either clofibrate or bezafibrate. The combination of increased nCEH and decreased ACAT could thus account for the reduced hepatic cholesteryl ester content.

Although liver nCEH has been shown to be activated *in vitro* by cAMP-dependent protein kinase, the maximum activation recorded was around 140% (25). This makes it unlikely that the increases in nCEH activity reported in the present work were due to phosphorylation of the enzyme, although such a covalent modification could make a contribution. Increases have been reported in a number of other cellular proteins, e.g., carnitine acyltransferase (26,27), fatty acid binding protein (28,29), and peroxisomal β-oxidation (30), following fibrate treatment of rats, and the extent of the increase in nCEH activity is comparable with these. The increases have been suggested to occur, *via* induction of the genes for these proteins, by activation of a group of specific transcription factors (31). It is probable that the enhanced nCEH activity can be ascribed to the same cause, although this would have to be ascertained by the appropriate investigation.

The increased nCEH activity may be a consequence of other changes in cellular metabolism resulting from fibrate treatment. There is a higher rate of peroxisomal (30) and mitochondrial (27) fatty acid oxidation in the livers of such animals, as well as an increase in the activity of acyl-CoA synthetase (32), both of which impose an enhanced requirement for long-chain fatty acids. This cannot be met by increased synthesis because the fibrates also cause inhibition of hepatic acetyl-CoA carboxylase (33), the rate-limiting enzyme in the synthesis of fatty acids. The increased hydrolysis of cholesteryl esters, combined with decreased esterification, may be a response to this increased demand for long-chain fatty acids.

In contrast to the large increases in nCEH activity, the much smaller changes in the activity of the cytosolic inhibitor of this enzyme (15,16), produced by bezafibrate, would not be expected to have major effects. Nevertheless, the reductions observed were such as to accentuate the effect of the changes in nCEH activity.

It is of interest to consider the clinical implications of the fibrate-induced changes in hepatic ACAT and nCEH activity. Hyperlipidemic patients treated with fibric acid derivatives exhibit a reduction in the circulating levels of VLDL which is due to a combination of increased catabolism, through stimulation of lipoprotein lipase activity (1,2), and a reduction in VLDL synthesis and secretion (3). The latter has been confirmed in studies using HepG2 cells (34). Although this has been suggested to be due to limitations in the supply of fatty acid substrate for VLDL secretion (3,35), incubation of HepG2 cells with fenofibrate also caused a reduction in cholesteryl esters as well as in triacylglycerols and free fatty acids (35).

Intracellular stores of cholesteryl ester have been shown to be necessary for VLDL secretion (36), and studies in which these stores have been depleted by inhibition of either HMG-CoA reductase (36) or ACAT (37) have revealed decreased triacylglycerol secretion. The serum triacylglycerol levels recorded in this study were in general

lower than literature values (26,38,39). This, and the lack of significant effect in the animals given fibrates, may result from the feeding regime where animals were given food at the start of the light period and sacrificed 24 h later. Nevertheless, similarly nonsignificant effects of fibrates on serum triacylglycerol levels have been reported by Stahlberg *et al.* (4). The activity of ACAT in human liver is unaffected by fibrate treatment (5) and bezafibrate failed to modulate HMG-CoA reductase activity either directly or indirectly in human mononuclear cells (40). If the reduction in VLDL secretion seen in patients, given fibric acid derivatives, and in fibrate-treated HepG2 cells is a consequence of a decrease in the cholesteryl ester content of the cells, our observations suggest that this could result from an increase in the nCEH activity of human liver similar to that occurring in rat liver.

Furthermore, treatment of hyperlipidemic patients with fibrates commonly causes an increase in the cholesterol content of bile, leading to supersaturation and the concomitant formation of gallstones (11,41). Although the mechanism of this hypersecretion of biliary cholesterol has not been fully explained, the results presented here may help to clarify the situation. There is considerable evidence that different pools of cholesterol exist within the hepatocyte and that specific pools control the secretion of cholesterol into bile (for a review, see Ref. 42). Inappropriate increases in the supply of cholesterol to such a pool has been shown to lead to enhanced biliary sterol secretion (43). The free cholesterol resulting from the combination of reduced ACAT and increased nCEH could also feed into such a pool leading to increased secretion of cholesterol into bile. Such an interrelationship between hepatic cholesterol esterification and biliary cholesterol output has been previously studied by Nervi *et al.* (44) and Stone *et al.* (45). They showed that inhibition of ACAT by various dietary and pharmacological manipulations was associated with enhanced secretion of cholesterol into bile. Continual removal of cholesterol to bile may then result in the reduction of cell cholesterol to the levels observed in this study.

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Liver, Serum and Adipose Tissue Fatty Acid Composition in Suckling Zucker Rats

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Young adult obese Zucker rats have altered tissue fatty acid (FA) composition. The present study was aimed at determining whether such changes were seen in either liver, serum or adipose tissue obtained from 17-day-old obese (fafa) rats in comparison to both homozygous (FaFa) and heterozygous (Fafa) lean rats. Body weights of obese pups (30.3 g) were significantly greater than those of homozygous lean rats (25.2 g) ($P < 0.05$). Liver weight and lipid content were similar in all groups. Inguinal fat pad weight and lipid content were greatest in obese pups (573 mg) followed by heterozygous lean pups (303 mg); homozygous lean pups (146 mg) had the lowest values. There were no differences among the groups in hepatic FA composition in either triacylglycerol (TG) or phospholipid fractions. Serum TG was similar among the groups, while serum phospholipid was greater ($P < 0.05$) in obese (269 mg/dL) than in homozygous lean pups (184 mg/dL); heterozygous lean pups had an intermediate value not significantly different from either homozygous group. On a percent basis, there were no differences in FA composition in either serum lipid fraction among the three groups. There were a number of significant differences in adipose tissue FA composition between the groups on a percent basis. The adipose tissue FA composition on a percent basis reflected that of maternal milk. The results indicate that suckling obese Zucker rats do not have tissue FA profiles that are characteristic of essential FA deficiency.

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Many metabolic abnormalities are seen in the genetically obese Zucker rat, including hyperinsulinemia (1) and hyperlipidemia (2,3). Excessive lipid synthesis in liver and adipose tissue has been documented (4–7), and cellular insulin resistance has been shown to develop as the rats age (8,9). Recent studies have been aimed at establishing a molecular basis for some of these effects (10–12), but the genetic lesion responsible for the obesity *per se* has not been identified.

Establishing the order of appearance of these metabolic disturbances in the Zucker rat will assist in delineating the defects that are responsible for the obesity. However, investigations on young, suckling rats have been limited because techniques for genotyping suckling rats have been lacking. Lavau and Bazin (13) described a relatively simple means for separating lean from obese pups at 16–17 d of age. This technique utilizes inguinal fat pad weights and fat pad-to-body weight ratios to distinguish suckling homozygous obese (fafa) pups from their heterozygous lean (Fafa) littermates when the pups were taken from litters with heterozygous lean (Fafa) mothers and homozygous obese (fafa) fathers. The suckling pups that became obese as adults had greater fat pad weights and pad-to-body weight ratios at 16–17 d of age. We have recently reported similar findings (14). The method allows for quick assessment of the pups' genotype and allows the use of animals at a time when the body weights of lean and obese pups are similar and the pups consume similar quantities of milk (15).

Milk intake has been reported to be similar for lean and obese pups (15). However, suckling obese pups have elevated adipose tissue lipid synthesis (14,16,17) and greater adipose tissue mass (13,14,16,17) compared to lean pups (14,16,17). Since polyunsaturated fatty acid (FA) intake is limited by dietary intake, one could hypothesize that altered tissue FA profiles may be an early characteristic of obesity. A number of studies (18–20) have previously shown that postweaning obese rats, compared to lean rats, shows altered FA composition indicative of early essential FA deficiency in several tissues.

In the present study, the FA composition of liver and serum triacylglycerol (TG) and phospholipid (PL) fractions and of inguinal adipose tissue total lipid were determined in 17-day-old rat pups. We used obese (fafa) pups and lean Zucker rats of two genotypes, FaFa and Fafa. This was done to determine if there was an effect of the "fa" gene in young lean rats, as other studies had shown that the presence of this gene in lean rats had some impact (14,21,22). The composition of mother's milk was also determined.

MATERIALS AND METHODS

Animals. Rats were obtained from a colony maintained at the Hormel Institute (Austin, MN). Rooms were temperature (21°C) and humidity (50%) controlled and maintained at a 12-h light/dark cycle. The study was approved by the University of Minnesota Animal Care and Use Committee. The Hormel Institute Animal Facility is accredited by the American Association for Accreditation of Laboratory Animal Care.

From 5 wk of age, the female rats designated to be mothers in this experiment were fed a low-fat diet consisting, on a g/100 g basis, of: cornstarch, 58; casein, 20.5; celufil, 9.0; sucrose, 5.0; salt mixture, 4.0; vitamin mixture, 1.0; methionine, 0.5; and corn oil, 2.0 (all components were from United States Biochemical Corporation, Cleveland, OH). FA composition of the diet on a percent basis was 14:0, 0.2; 16:0, 12.1; 18:0, 2.3; 18:1ω9, 26.4; 18:2ω6, 57.4; 18:3ω3, 1.0; 20:0, 0.3; and 21:0, 0.3. Upon reaching 200 g body weight and/or 10 wk of age, female rats were bred to male rats. The female rats were housed in plastic cages with wood shavings throughout mating, pregnancy and lactation. Specially designed food cup holders were used to prevent the pups from eating their mother's food.

Two types of breeding crosses were used. Homozygous (FaFa) lean male and female rats were bred to provide homozygous (FaFa) lean pups, and homozygous (fafa) obese

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Abbreviations: FA, fatty acid; FID, flame-ionization detector; PL, phospholipids; TG, triacylglycerol.

male rats were mated with heterozygous (Fafa) lean female rats to provide heterozygous (Fafa) lean and homozygous (fafa) obese pups.

At 17 d of age, pups were killed by decapitation, and blood was collected for serum preparation. Livers as well as left and right inguinal fat depots were removed and weighed. When pups were obtained from the obese male/heterozygous lean female crosses, fat depot weight and fat pad-to-body weight ratio were used to identify lean and obese pups. Liver and adipose tissue were processed as described below. Each mother rat was separated from her pups for one hour and then injected intraperitoneally with 2 IU of oxytocin. Under mild diethylether anesthesia, approximately 2 mL of milk were expressed by gentle manipulation of teats with thumb and forefinger. Milk droplets were collected using mild suction.

Adipose tissue total lipid extraction. Total adipose tissue lipid was extracted using a modification of the method by Dole and Meinertz (23). The tissue (100–700 mg) was homogenized with 5 mL Dole's reagent (isopropanol/hexane/1 N H_2SO_4, 40:10:1, by vol). Next, 3 mL water and 3 mL hexane were added, and the tube was shaken for 30 s. The layers were allowed to separate for 15 min, and the hexane phase was removed. The lower phase was reextracted twice with 3 mL of hexane. After the hexane had been evaporated with N_2, the total lipid was dissolved in a known volume of methylene chloride, and aliquots were taken for lipid determination.

Liver tissue total lipid extraction. Total liver lipids were extracted using the method developed by Phillips and Privett (24). Tissue (0.5 g) and 5 mL of hot acetic acid (0.05 N) were heated in a water bath (95°C) for 30 min. The sample was homogenized and centrifuged at 12,000 rpm for 15 min. The acetic acid was decanted and the pellet resuspended in 5 mL acetic acid (0.05 N) and centrifuged, and the acetic acid phase was decanted. The pellet was transferred to a 40-mL glass tube containing 12.5 mL methanol, then 12.5 mL of chloroform was added before the mixture was homogenized for 30 s. After standing for 15 min at room temperature, the sample was centrifuged for 5 min at 3,000 rpm. The chloroform/methanol extract was decanted, and the pellet was reextracted first with 20 mL of chloroform/methanol (1:1, vol/vol) and then with 20 mL of chloroform/methanol (1:2, vol/vol). The combined extracts were evaporated under vacuum. The dried lipid extract was dissolved in a known volume of methylene chloride, and aliquots were taken for lipid determination.

Milk and serum total lipid extraction. Each sample (200 μL) was added dropwise to stirred ice-cold methanol (3.4 mL). The sample was placed in an ice bath for 10 min, followed by addition of 3.4 mL of ice-cold chloroform. After an additional 10 min, 6.8 mL of ice-cold chloroform/methanol (1:1, vol/vol) was added, and the sample was centrifuged for 3 min at 3,000 rpm. The lipid-containing chloroform/methanol layer was decanted into a 15-mL screw-cap tube and evaporated under N_2. The lipid extract was dissolved in a known volume of methylene chloride, and aliquots were taken for lipid determination.

Isolation of serum TG and PL. The total lipid extract (0.5–5 mg) was spotted onto a thin-layer chromatographic plate (silica gel H, 0.3 mm layer). The plate was developed in a paper-lined chamber using hexane/diethyl ether/acetic acid (200:50:2, by vol) as solvent. After development, the TG and PL zones were identified under an ultraviolet lamp after spraying the plates with 0.2% 2,7-dichlorofluorescein in methanol and drying with N_2. The TG and PL zones were scraped into glass screw-cap tubes, and 0.5 mL methylene chloride was added. Samples were transesterified as described below.

FA analysis. The isolated TG and PL fractions and the fat tissue lipids were transesterified by adding 2 mL of 3N HCl/methanol reagent, flushing with N_2, and heating at 90°C for 1 h for the TG and for 2 h for the PL fractions. The tubes were cooled, and an equal volume of water was added. The methyl esters were extracted twice with 5 mL hexane. The hexane was evaporated, and the esters were dissolved in methylene chloride. Samples were analyzed using a Hewlett-Packard 5840A gas–liquid chromatograph equipped with a 30 m × 0.26 mm DB225 (0.25 μm film) capillary column (J&W Scientific, Folsom, CA) and flame-ionization detector (FID) using helium as the carrier gas. The temperature was programmed from 160 to 225°C at 2°C/min.

Statistics. Data are presented as means ± SEM. FA data are given in area percent unless otherwise noted. Five pups were used in each group. Statistical comparisons were made by one-way analysis of variance followed by Least Significant Difference test to determine significance between specific groups. A total of 27 FA between 10:0 and 24:1ω9 were analyzed. For values obtained that were greater than 0.5%, all data are presented whether or not there were significant differences. For values less than 0.5%, only those data are presented for which statistically significant differences were seen.

Fourteen samples of mother's milk were obtained (seven homozygous and seven heterozygous females). There were no differences in fatty acid composition of the milk between the two genotypes; therefore, the results for these two groups were combined.

RESULTS

At 17 d of age, obese pups weighed significantly more than homozygous lean pups (Table 1). The heterozygous lean pups had an intermediate body weight that was not significantly different from that of either of the other two groups. Liver weights and amounts of liver lipids were similar among the three groups (Table 1). Inguinal fat pad weight was greatest in obese pups followed by that of heterozygous lean pups (Table 1); homozygous lean pups had the lightest fat pads. Fat pad lipids followed a similar pattern. The fat pad-to-body weight ratio was higher in obese compared to lean pups. Serum TG concentrations were similar in all three groups. Serum PL concentrations were greater in obese compared to homozygous lean rats, and heterozygous lean pups had an intermediate value not significantly different from either homozygous group.

As can be seen in Table 2, there were minor differences in hepatic FA composition among the three groups in both the TG and PL fractions when results were expressed on a percent basis. No significant differences were seen when

TABLE 1

Body Weight, Liver Weight, Liver Lipid Weight, Inguinal Fat Pad Weight, Fat-to-Body Weight Ratio and Serum Lipid Levels in 17-day-old Zucker Rat Pups[a]

	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese
Body weight (g)	$25.2^{b} \pm 0.6$	$27.7^{b,c} \pm 2.3$	$30.3^{c} \pm 1.0$
Liver weight (mg)	$764^{b} \pm 14$	$888^{b} \pm 77$	$895^{b} \pm 48$
Liver lipid weight (mg)	$30.9^{b} \pm 0.8$	$35.7^{b} \pm 3.4$	$36.8^{b} \pm 2.0$
Inguinal pad weight (mg)	$146^{b} \pm 6$	$303^{c} \pm 33$	$537^{d} \pm 48$
Fat pad lipid weight (mg)	$83^{b} \pm 2$	$211^{c} \pm 27$	$408^{d} \pm 41$
Fat pad-to-body weight	$0.0058^{b} \pm 0.0002$	$0.0075^{b} \pm 0.0005$	$0.0144^{c} \pm 0.0015$
Serum triacylglycerol (mg/dL)	$163^{b} \pm 5$	$180^{b} \pm 21$	$203^{b} \pm 15$
Serum phospholipid (mg/dL)	$184^{b} \pm 5$	$229^{b,c} \pm 27$	$269^{c} \pm 20$

[a]Values are mean ± SEM; n = 5 per group.
[b,c,d]Across a row, numbers with different superscripts are significantly different; $P < 0.05$.

FA were grouped by saturation level. Although no statistical comparisons were made, it can be seen that there were distinct differences in FA composition between the two lipid classes. Since liver weights and lipid contents were similar, there were no differences among the groups when absolute (mg/g) amounts of individual FA were determined (data not shown).

On a percent basis, there were essentially no differences in either serum TG or PL FA profiles among the three groups of 17-day-old rat pups (Table 3). As in the liver, there were differences between the two lipid fractions with respect to the distribution of the FA by the degree of unsaturation. As shown in Table 1, obese pups did have significantly higher serum PL concentrations compared to

TABLE 2

Hepatic Triacylglycerol and Phospholipid Fatty Acid Composition by Percent in 17-day-old Zucker Rat Pups (area %)[a]

	Triacylglycerol			Phospholipid		
	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese
12:0	$1.4^{b} \pm 0.1$	$1.7^{b} \pm 0.4$	$1.3^{b} \pm 0.4$	$0.1^{b} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{b} \pm 0.0$
14:0	$5.1^{b} \pm 0.1$	$6.9^{b} \pm 0.4$	$6.7^{b} \pm 0.7$	$0.9^{b} \pm 0.0$	$1.3^{b} \pm 0.1$	$1.3^{b} \pm 0.0$
16:0	$31.2^{b} \pm 0.3$	$31.6^{b} \pm 0.7$	$33.8^{b} \pm 0.9$	$23.7^{b} \pm 0.6$	$25.1^{b} \pm 0.5$	$25.2^{b} \pm 0.4$
16:1ω9	$0.3^{b} \pm 0.0$	$0.4^{b} \pm 0.0$	$0.5^{b} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{b} \pm 0.0$
16:1ω7	$0.9^{b} \pm 0.0$	$1.1^{b} \pm 0.0$	$1.3^{b} \pm 0.1$	$0.2^{b} \pm 0.0$	$0.2^{b} \pm 0.0$	$0.3^{b} \pm 0.0$
17:0	$0.2^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.2^{b} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.2^{b,c} \pm 0.0$
18:0	$4.5^{b} \pm 0.1$	$4.1^{b} \pm 0.2$	$4.9^{b} \pm 0.3$	$20.8^{b} \pm 0.2$	$20.0^{b} \pm 0.3$	$19.2^{b} \pm 0.3$
18:1ω9	$24.2^{b} \pm 0.2$	$21.1^{b} \pm 0.6$	$22.4^{b} \pm 1.0$	$3.9^{b} \pm 0.2$	$3.1^{b} \pm 0.1$	$3.3^{b} \pm 0.2$
18:1ω7	$1.7^{b} \pm 0.0$	$1.8^{b} \pm 0.0$	$2.1^{c} \pm 0.1$	$0.9^{b} \pm 0.1$	$0.9^{b} \pm 0.0$	$1.0^{b} \pm 0.0$
18:2ω6	$16.9^{b} \pm 0.1$	$17.0^{b} \pm 0.6$	$15.8^{b} \pm 0.6$	$7.1^{b} \pm 0.1$	$7.4^{b} \pm 0.2$	$8.2^{b} \pm 0.4$
18:3ω6	$0.2^{b} \pm 0.0$	$0.5^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.1^{b} \pm 0.0$	$0.1^{b} \pm 0.0$
18:3ω3	$0.1^{b} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.1^{b,c} \pm 0.0$	$0.1^{c} \pm 0.0$	$<0.1^{b} \pm 0.0$	$<0.1^{b} \pm 0.0$
20:2ω6	$0.3^{b} \pm 0.0$	$0.6^{b} \pm 0.0$	$0.6^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.3^{b} \pm 0.0$
20:3ω9	$1.0^{b} \pm 0.1$	$0.8^{b} \pm 0.1$	$0.7^{b} \pm 0.1$	$0.2^{b} \pm 0.1$	$0.1^{b} \pm 0.1$	$0.2^{b} \pm 0.1$
20:3ω6	$0.7^{b} \pm 0.0$	$0.6^{b} \pm 0.0$	$0.5^{b} \pm 0.1$	$0.6^{b} \pm 0.0$	$0.6^{b} \pm 0.0$	$0.8^{b} \pm 0.1$
20:4ω6	$5.1^{b} \pm 0.1$	$5.3^{b} \pm 0.6$	$4.0^{b} \pm 0.6$	$26.2^{b} \pm 0.4$	$25.2^{b} \pm 0.2$	$25.8^{b} \pm 0.3$
22:4ω6	$2.7^{b} \pm 0.1$	$2.7^{b} \pm 0.2$	$2.2^{b} \pm 0.3$	$1.8^{b} \pm 0.1$	$1.9^{b} \pm 0.1$	$1.9^{b} \pm 0.1$
22:5ω6	$3.3^{c} \pm 0.0$	$2.4^{b} \pm 0.2$	$2.0^{b} \pm 0.2$	$7.8^{b} \pm 0.2$	$7.4^{b} \pm 0.2$	$7.0^{b} \pm 0.2$
22:5ω3	$0.4^{b} \pm 0.0$	$0.4^{b} \pm 0.0$	$0.2^{b} \pm 0.0$	$0.8^{b} \pm 0.0$	$0.9^{b} \pm 0.0$	$0.9^{b} \pm 0.0$
22:6ω3	$0.2^{b} \pm 0.0$	$0.3^{b} \pm 0.0$	$0.2^{b} \pm 0.0$	$4.1^{b} \pm 0.2$	$4.2^{b} \pm 0.2$	$3.5^{b} \pm 0.2$
24:0	<0.1	<0.1	<0.1	$0.3^{b} \pm 0.0$	$0.4^{b,c} \pm 0.0$	$0.4^{c} \pm 0.0$
Sum of:						
Saturated	$42.5^{b} \pm 0.3$	$45.1^{b} \pm 1.2$	$47.1^{b} \pm 1.0$	$46.1^{b} \pm 0.5$	$46.9^{b} \pm 0.2$	$46.4^{b} \pm 0.2$
Monounsaturated	$27.6^{b} \pm 0.3$	$24.9^{b} \pm 0.6$	$26.9^{b} \pm 1.2$	$5.2^{b} \pm 0.2$	$4.7^{b} \pm 0.1$	$5.0^{b} \pm 0.3$
Polyunsaturated	$31.2^{b} \pm 0.4$	$31.2^{b} \pm 1.6$	$27.1^{b} \pm 1.7$	$48.9^{b} \pm 0.6$	$48.1^{b} \pm 0.3$	$48.8^{b} \pm 0.3$

[a]Values are means of five rats/group ± SEM. A total of 27 fatty acids between 10:0 and 24:1ω9 were assayed. Results were included when at least one experimental group had a value of 0.5% or greater for either the triacylglycerol or phospholipid fraction or when values were significantly different. Fatty acids with values less than 0.5% where there were no significant differences were 10:0, 14:1ω5, 15:0, 20:1ω9, 20:5ω3, and 24:1ω9.
[b,c]Values with different superscripts are significantly different at $P < 0.05$ for that lipid fraction.

TABLE 3

Serum Triacylglycerol and Phospholipid Fatty Acid Composition by Percent in 17-day-old Zucker Rat Pups (area %)[a]

	Triacylglycerol			Phospholipid		
	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese
10:0	0.3[b] ± 0.2	0.1[b] ± 0.0	0.5[b] ± 0.3	0.0[b] ± 0.0	0.0[b] ± 0.0	0.0[b] ± 0.0
12:0	4.3[b] ± 1.0	4.4[b] ± 0.6	5.7[b] ± 1.0	0.1[b] ± 0.0	0.2[b] ± 0.0	0.2[b] ± 0.0
14:0	11.2[b] ± 0.6	12.3[b] ± 0.4	12.0[b] ± 0.7	1.3[b] ± 0.1	1.5[c] ± 0.1	1.4[b] ± 0.0
15:0	0.3[b,c] ± 0.0	0.4[c] ± 0.1	0.2[b] ± 0.1	0.1[b] ± 0.0	0.1[b] ± 0.0	0.1[b] ± 0.0
16:0	36.5[b] ± 0.4	33.1[b] ± 1.1	32.8[b] ± 1.0	26.9[b] ± 0.2	25.5[b] ± 0.2	25.2[b] ± 0.2
16:1ω9	0.4[b] ± 0.0	1.1[c] ± 0.1	1.0[c] ± 0.2	0.2[b] ± 0.0	0.1[b] ± 0.0	0.1[b] ± 0.0
16:1ω7	1.1[b] ± 0.0	1.3[b] ± 0.1	1.3[b] ± 0.1	0.2[b] ± 0.0	0.3[b] ± 0.0	0.3[b] ± 0.0
17:0	0.4[c] ± 0.0	0.2[b] ± 0.0	0.1[b] ± 0.0	0.3[b] ± 0.0	0.3[b] ± 0.0	0.2[b] ± 0.0
18:0	4.7[b] ± 0.4	3.6[b] ± 0.1	4.0[b] ± 0.5	16.8[b] ± 0.1	17.3[b] ± 0.2	16.8[b] ± 0.1
18:1ω9	16.6[b] ± 0.7	17.6[b] ± 0.5	17.3[b] ± 0.8	3.6[c] ± 0.0	3.7[b] ± 0.2	3.9[b] ± 0.2
18:1ω7	1.2[b] ± 0.0	1.4[b] ± 0.1	1.4[b] ± 0.1	0.9[b] ± 0.0	0.9[b] ± 0.0	1.0[b] ± 0.0
18:2ω6	12.6[b] ± 0.5	12.4[b] ± 0.3	11.8[b] ± 0.8	25.2[b] ± 0.2	24.2[b] ± 0.9	24.9[b] ± 0.7
20:2ω6	0.3[b] ± 0.0	0.4[b] ± 0.1	0.5[b] ± 0.0	0.3[b] ± 0.0	0.4[b] ± 0.0	0.4[b] ± 0.0
20:3ω9	0.9[b] ± 0.4	1.0[b] ± 0.4	1.2[b] ± 0.1	0.2[b] ± 0.1	0.2[b] ± 0.1	0.2[b] ± 0.1
20:3ω6	0.3[b] ± 0.0	0.3[b] ± 0.0	0.3[b] ± 0.0	0.7[b] ± 0.0	1.0[c] ± 0.1	1.1[c] ± 0.1
20:4ω6	4.9[b] ± 0.4	6.7[b] ± 0.8	7.0[b] ± 1.2	15.2[b] ± 0.2	17.0[b] ± 0.7	15.9[b] ± 0.7
22:4ω6	1.1[b] ± 0.1	1.4[b] ± 0.2	1.4[b] ± 0.2	1.1[b] ± 0.0	1.1[b] ± 0.0	1.2[b] ± 0.0
22:5ω6	1.9[c] ± 0.1	1.8[b] ± 0.2	1.4[b] ± 0.4	3.4[c] ± 0.0	2.9[b] ± 0.2	3.0[b] ± 0.0
22:5ω3	0.3[b] ± 0.0	0.3[b] ± 0.0	0.2[b] ± 0.0	0.6[b] ± 0.0	0.6[b] ± 0.0	0.7[b] ± 0.0
22:6ω3	0.2[b] ± 0.0	0.2[b] ± 0.0	0.2[b] ± 0.0	1.9[b] ± 0.0	2.0[b] ± 0.1	1.7[b] ± 0.1
24:0	0.1[b] ± 0.0	0.1[b] ± 0.0	0.0[b] ± 0.0	0.5[b] ± 0.0	0.5[b] ± 0.0	0.5[b] ± 0.0
24:1ω9	0.2[b] ± 0.0	0.0[b] ± 0.0	0.0[b] ± 0.0	0.5[b] ± 0.0	0.4[b] ± 0.0	0.5[b] ± 0.0
Sum of:						
Saturated	57.7[b] ± 1.6	54.2[b] ± 1.1	55.4[b] ± 2.3	46.1[b] ± 0.2	45.2[b] ± 0.3	44.8[b] ± 0.1
Monounsaturated	19.7[b] ± 0.7	21.7[b] ± 0.5	21.2[b] ± 1.0	5.3[b] ± 0.1	5.4[b] ± 0.2	5.9[b] ± 0.2
Polyunsaturated	23.6[b] ± 0.9	25.4[b] ± 1.4	24.7[b] ± 2.5	48.8[b] ± 0.2	49.6[b] ± 0.2	49.4[b] ± 0.2

[a]Values are means of five rats/group ± SEM. A total of 27 fatty acids between 10:0 and 24:1ω9 were assayed. Results were included when at least one experimental group had a value of 0.5% or greater for either the triacylglycerol or phospholipid fraction or when values were significantly different. Fatty acids with values less than 0.5% where there were no significant differences were 14:1ω5, 18:3ω6, 18:3ω3, 20:1ω9, and 20:5ω3.

[b,c]Values with different superscripts are significantly different at $P < 0.05$ for that lipid fraction.

homozygous lean pups, and also showed a trend toward higher serum TG levels. When serum FA composition was expressed as mg/dL, significant differences were noted between obese and homozygous lean pups for several FA in the TG fraction, but there were no differences in the sums for saturated, monounsaturated or polyunsaturated FA (results not shown). In the PL fraction, most levels of individual FA calculated as mg/dL were significantly higher in obese than in homozygous lean rats (results not shown). Of particular interest is that 18:2ω6 and 20:4ω6 levels were 50% higher in obese rats compared to homozygous lean rats. The heterozygous lean rats tended to have intermediate values for most FA that were not consistently and statistically different from those of the other two groups.

Adipose tissue FA composition as a percent of total lipids is presented in Table 4. Compared to either serum or liver, fewer FA were identified at >0.5% in adipose tissue. However, a number of statistically significant differences were found for these FA present in low amounts. In general, obese and heterozygous lean rats had significantly higher values for ω6 and ω3 FA when they were present at low levels. However, 18:2ω6 values were similar in all three groups. Obese rats had slightly higher levels of several medium chain FA, particularly when compared to homozygous lean rats. Both homozygous and heterozygous lean rats had higher levels of 18:0, 18:1ω9 and 18:1ω7 in adipose tissue than did obese rats. There were no differences among the groups for the sum of saturated FA. The summed monounsaturated FA levels were highest in homozygous lean rats, intermediate in heterozygous lean rats, and lowest in obese rats. Heterozygous lean rats had higher total polyunsaturated FA levels than did homozygous lean rats, and obese rats had an intermediate value that was not significantly different from that of the two lean groups. In general, significant differences found among the groups were minor. The composition of mother's milk is also included in Table 4. Adipose tissue FA composition on a percent basis was similar to that of maternal milk obtained on the seventeenth day of lactation. As expected due to differences in fat pad weights, large significant differences in the amounts of FA were found among the three groups when FA composition was presented on a per depot basis (results not shown). For all FA,

TABLE 4

Adipose Tissue Fatty Acid Composition by Percent of 17-day-old Rat Pups and Rat Milk at 17 Days of Lactation (area %)[a]

	Homozygous (FaFa) lean	Heterozygous (Fafa) lean	Homozygous (fafa) obese	Mother's milk
10:0	$1.8^{b} \pm 0.2$	$2.7^{c} \pm 0.3$	$3.2^{c} \pm 0.3$	0.5 ± 0.2
12:0	$8.4^{b} \pm 0.3$	$10.4^{c} \pm 0.1$	$11.7^{d} \pm 0.3$	9.7 ± 1.0
14:0	$14.0^{b} \pm 0.1$	$14.5^{b} \pm 0.4$	$15.6^{c} \pm 0.1$	17.2 ± 1.21
14:1ω5	$0.1^{b} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.3^{d} \pm 0.0$	n.d.
16:0	$37.0^{c} \pm 0.2$	$32.8^{b} \pm 0.8$	$32.6^{b} \pm 0.7$	35.6 ± 1.2
16:1ω7	$2.4^{b} \pm 0.1$	$3.2^{c} \pm 0.1$	$3.9^{d} \pm 0.1$	2.2 ± 0.2
18:0	$3.6^{d} \pm 0.1$	$2.8^{c} \pm 0.1$	$2.2^{b} \pm 0.1$	2.9 ± 0.4
18:1ω9	$20.0^{d} \pm 0.2$	$17.5^{c} \pm 0.3$	$16.1^{b} \pm 0.2$	17.4 ± 1.9
18:1ω7	$2.0^{c} \pm 0.1$	$1.9^{c} \pm 0.1$	$1.6^{b} \pm 0.1$	1.5 ± 0.2
18:2ω6	$8.9^{b} \pm 0.1$	$9.8^{b} \pm 0.5$	$9.0^{b} \pm 0.4$	9.3 ± 0.2
18:3ω6	$0.1^{b} \pm 0.0$	$0.2^{c} \pm 0.0$	$0.2^{b,c} \pm 0.0$	0.2 ± 0.0
18:3ω3	$<0.1^{b}$	$0.2^{c} \pm 0.0$	$0.2^{c} \pm 0.0$	0.3 ± 0.0
20:2ω6	$0.3^{b} \pm 0.1$	$0.5^{b} \pm 0.1$	$0.4^{b} \pm 0.1$	0.3 ± 0.1
20:3ω6	$0.2^{b} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.3^{c} \pm 0.0$	0.2 ± 0.0
20:4ω6	$0.7^{b} \pm 0.1$	$1.2^{d} \pm 0.1$	$1.0^{c} \pm 0.1$	0.8 ± 0.1
22:4ω6	$0.2^{b} \pm 0.0$	$0.5^{c} \pm 0.0$	$0.4^{c} \pm 0.0$	0.3 ± 0.1
22:5ω6	$0.2^{b} \pm 0.0$	$0.3^{c} \pm 0.0$	$0.3^{c} \pm 0.0$	0.1 ± 0.1
22:5ω3	$<0.1^{b}$	$0.1^{c} \pm 0.0$	$0.1^{c} \pm 0.0$	n.d.
22:6ω3	$<0.1^{b}$	$0.1^{c} \pm 0.0$	$0.1^{c} \pm 0.0$	n.d.
Sum of:				
Saturated	$65.2^{b} \pm 0.3$	$63.6^{b} \pm 0.9$	$65.7^{b} \pm 0.3$	66.4 ± 2.8
Monounsaturated	$25.0^{d} \pm 0.2$	$23.4^{c} \pm 0.2$	$22.4^{b} \pm 0.2$	21.8 ± 2.4
Polyunsaturated	$10.8^{b} \pm 0.1$	$13.3^{c} \pm 0.7$	$12.1^{b,c} \pm 0.40$	11.6 ± 0.5

[a]Values are means of five rats/group ± SEM (except for mother's milk n = 14). A total of 27 fatty acids between 10:0 and 24:1ω9 fatty acids were assayed. Results were included when at least one experimental group had a value of 0.5% or greater or when values were significantly different. Fatty acids with values less than 0.5% where there were no significant differences were 15:0, 16:1ω9, 17:0, 20:1ω9, 20:3ω9, 20:5ω3, 24:0, and 24:1ω9; n.d., not detected.

[b,c,d]Values with different superscripts are significantly different at $P < 0.05$.

obese Zucker rats had the highest levels, heterozygous lean rats had intermediate levels, and homozygous lean rats had the lowest levels. This correlation was also seen when FA were grouped by saturation level (results not shown).

DISCUSSION

In general, the results indicate that suckling obese Zucker rats do not manifest patterns of altered polyunsaturated FA levels in either serum, liver or adipose tissue. These findings contrast with previously reported studies in which tissue FA compositions of older obese and lean Zucker rats were compared (18–20). Earlier, Wahle (19) had reported that adipose tissue from 90–120-day-old Zucker obese rats had higher levels of 16:0 and 16:1, similar amounts of 18:0 and 18:1, but dramatically lower 18:2 levels than did adipose tissue from lean rats. Results for liver were similar except that 18:0 tended to be lower in obese rats than in lean rats. The general conclusion was that monounsaturated FA replaced/substituted for the polyunsaturated FA the levels of which were limited by dietary content and intake in the obese rats.

In a more extensive study, Guesnet *et al.* (18) evaluated more tissues and identified more FA in PL using three-month-old rats. Few differences in FA composition in tissues related to the nervous system were reported between lean and obese rats. However, in serum, liver, kidney and heart, n-6 polyunsaturated FA were lower in the tissues of obese rats than in lean rats, except for the liver, where they were similar.

The general conclusion drawn by several investigators (18,25) has been that obese rats have decreased Δ5 desaturase activity. However, Phinney *et al.* (26) found that supplementing obese Zucker rats with black currant seed oil, which is rich in γ-linolenic acid (18:3ω6), elevated 20:4n-6 tissue levels. This finding as well as the fact that products of the desaturase/elongase pathways were found in nonsupplemented obese rats led to the conclusion that the Δ6 and Δ5 desaturase systems were capable of producing sufficient amounts of product. This led to the hypothesis (26) that there is a loss of substrate specificity for 18:2ω6 and/or that there is enhanced catabolism of 20:4ω6 in obese rats.

In young obese suckling rats, however, we did not find evidence of an altered polyunsaturated FA pattern that would suggest a deficient state. In fact, obese rats had identical FA patterns when compared to lean rats when the results are presented on a percent basis. In agreement with this, Guerre-Millo *et al.* (27) recently reported on the

FA composition of PL of adipocyte plasma membranes from 30-day-old lean and obese Zucker rats. The authors found no differences between lean and obese rats except for a significant increase in 22:6n-3 in obese rats.

When results for adipose tissue FA composition were calculated on a depot basis, obese rats had five times more 18:2ω6 and almost seven times more 20:4ω6 than did homozygous lean rats. Clearly at 17 d of age, obese Zucker rats did not have lowered polyunsaturated FA tissue levels compared to lean pups. This would indicate that an adequate supply of these nutrients can be supplied by mother's milk. Milk fat content in the present study was 12.87 ± 0.59 (mean ± SEM g/dL). This value agrees with previously published data (15,28–30), and the FA composition of the milk agrees, in general, with results obtained on rats fed standard type diets (31,32).

In summary, (i) in suckling obese rats there was no evidence of polyunsaturated FA deficiency in liver, adipose tissue or serum, and (ii) adipose tissue FA composition reflected milk FA composition. Thus, the changes in polyunsaturated FA metabolism in Zucker rats must occur postweaning and are not involved in the early stages of obesity.

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Composition and Structure of Triacylglycerols in Brown Adipose Tissue of Rats Fed Fish Oil

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This study examines the incorporation of highly unsaturated n-3 fatty acids (HUFA) into triacylglycerols (TAG) of brown adipose tissue (BAT), and their effect on the positional distribution of saturated (SFA) and of unsaturated (UFA) 16- or 18-carbon fatty acids. To this end, rats were fed a fish oil diet for up to four weeks. The stereospecific analysis of TAG was based on generation of *sn*-1,2- and *sn*-2,3-acylglycerols by Grignard degradation, followed by synthesis of phosphatidic acid and specific hydrolysis with phospholipase A_2. From the end of the first week of fish oil feeding, a steady-state in the fatty acid composition of TAG in BAT was reached. HUFA concentration increased 30-fold, mainly at the expense of n-9 UFA and of SFA. The amount of SFA decreased selectively at position 3, where these fatty acids were progressively replaced by n-3 HUFA. By contrast, the amount of UFA decreased at all positions, and their positional distribution was not affected. About 60% of HUFA was incorporated at position 3. Nearly twice as much 22:6n-3 was incorporated into TAG than had been previously observed in white adipose tissue (WAT) [Leray, C., Raclot, T., and Groscolas, R. (1993)*Lipids 28*, 279–284]. At the steady-state, the distribution of HUFA was characterized by high proportions of 22:6n-3 and 20:5n-3 in position 3. Moreover, in each position of TAG, a steady level was reached rapidly (within 1 wk). It is concluded that, during fish-oil feeding, fatty acids in TAG of BAT show characteristic time-course changes that lead to a characteristic composition and a tissue-specific positional distribution. This suggests that adipose tissue has its own specificity in controlling the build-up of TAG stores, which is likely to be regulated by the specificity of acylating enzymes as well as molecular rearrangements.
***Lipids 29*, 759–764 (1994).**

Fatty acids are stored mainly as triacylglycerols (TAG) in adipose tissues where they comprise from about 50% (brown adipose tissue, BAT) to 90% (white adipose tissue, WAT) of tissue weight. Whereas the overall mechanisms of synthesis and degradation of TAG have been adequately described (1), the finer aspects of metabolic regulation at the molecular level are only poorly understood. In particular, only limited information is available on the control of the fatty acid distribution between the three sites of the glycerol moiety. Several reports (reviewed in Ref. 1) have provided evidence that esterifying enzymes tend to place the saturated fatty acids (SFA) at the *sn*-1 position of triacylglycerols, the unsaturated 16- and 18-carbon fatty acids (UFA) at the *sn*-2 position, and the highly unsaturated 20- and 22-carbon fatty acids (HUFA) at the *sn*-3 position. The 20- and 22-carbon HUFA of the n-3 series are abundant in fish oil and have been shown to be stored after ingestion by rats in adipose tissue (2–4). HUFA have been shown to play important roles as antagonists in eicosanoid biosynthesis (5) and are known to prevent or to alleviate various pathological conditions (6). Recently, HUFA have been shown to limit the hypertrophy of abdominal depots in growing rats fed high-fat diets (7). It is thus of interest to gain a better understanding of the action of exogenous n-3 HUFA on storage tissues and, conversely, of the influence of these fatty acids on the structure of the TAG stored. The two types of adipose tissue found in mammals, the WAT and the BAT, are well known for their physiologically distinct functions. Whereas WAT is the reserve of fat energy for other tissues (8), BAT is the main site of nonshivering thermogenesis (9) with its stored fatty acids being mainly oxidized in mitochondria. Because of its distinct size and metabolic activity, BAT may be expected to accumulate TAG by processes and with time-dependencies different from those in WAT (2). Until now, this aspect of TAG storage has not been examined in BAT.

The purpose of the present study was to examine the time-course changes of the quantitative and stereospecific incorporation of n-3 HUFA into TAG of BAT in rats fed for up to four weeks a diet supplemented with fish oil. Concurrently, the stereospecific distribution of the other stored fatty acids, both of exogenous and endogenous origin, was also investigated. This made it possible to compare the fate of the fatty acids in the two types of adipose tissue.

MATERIALS AND METHODS

Animals and diet. Thirty-five male Wistar rats, individually housed under a 12-h light/dark cycle at 25°C, were fed a basal diet (AO, Usine d'Alimentation Rationnelle, Villemoisson, France) containing 3% total lipids by weight. The small amount of phospholipids present in this diet (17% of the total lipid moiety) had a fatty acid composition similar to that of TAG. When animals reached 225 g mean live body weight, they were given *ad libitum* a semisynthetic diet supplemented with fish oil (MaxEPA; R.P. Scherer, Beinheim, France; 19% in the diet). The fatty acid composition of the fish oil diet is given in Table 1. The dietary treatment used here was the same as that described previously (2).

Extraction and analysis of lipids. All animals were killed by cervical dislocation at 9 a.m. Five rats fed the basal diet were killed at the onset of the experimental feeding period. Groups of animals fed the fish oil diet were killed after feeding periods of 1 and 3 d, and 1, 2, 3 and 4 wk (five rats in each group). BAT samples were immediately extracted (10), and TAG were isolated by thin-layer chromatography using hexane/diethyl ether/acetic acid

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Abbreviations: BAT, brown adipose tissue; HUFA, 20- and 22-highly unsaturated fatty acids; SFA, saturated fatty acids; TAG, triacylglycerols; UFA, 16- and 18-unsaturated fatty acids; WAT, white adipose tissue.

TABLE 1

Fatty Acid Composition of Triacylglycerols in Fish Oil Diet and in Brown Adipose Tissue from Rats Fed This Diet[a]

Fatty acid	Fish oil diet	Brown adipose tissue			
		0 d	3 d	1 wk	4 wk
14:0	8.6	3.7 ± 0.1^{b}	4.7 ± 0.1^{c}	6.0 ± 0.2^{d}	5.7 ± 0.2^{d}
16:0	21.1	34.4 ± 0.9^{b}	29.8 ± 1.3^{c}	$30.5 \pm 2.4^{b,c,d}$	25.9 ± 0.3^{d}
18:0	3.7	10.1 ± 0.5^{b}	7.4 ± 0.1^{c}	7.9 ± 0.6^{c}	6.4 ± 0.5^{c}
18:1n-9	10.3	31.1 ± 0.7^{b}	26.0 ± 1.3^{c}	16.7 ± 0.9^{d}	$20.8 \pm 2.2^{c,d}$
20:1n-9	1.5	0.4 ± 0.1^{b}	$0.7 \pm 0.1^{b,c}$	$1.0 \pm 0.1^{c,d}$	1.3 ± 0.1^{d}
16:1n-7	10.0	6.0 ± 0.3^{b}	7.3 ± 0.5^{b}	6.5 ± 0.2^{b}	7.2 ± 0.6^{b}
18:1n-7	3.6	2.0 ± 0.1^{b}	$2.5 \pm 0.3^{b,c}$	3.3 ± 0.1^{c}	3.7 ± 0.1^{d}
18:2n-6	5.4	10.5 ± 0.3^{b}	$10.2 \pm 1.5^{b,c}$	8.6 ± 0.6^{c}	8.8 ± 0.2^{c}
18:4n-3	3.6	—	0.8 ± 0.1^{b}	1.2 ± 0.1^{c}	1.3 ± 0.1^{c}
20:5n-3	15.9	—	3.0 ± 0.3^{b}	4.7 ± 0.4^{c}	4.8 ± 0.4^{c}
22:5n-3	1.7	—	1.0 ± 0.1^{b}	2.0 ± 0.2^{c}	2.1 ± 0.3^{c}
22:6n-3	8.4	—	3.3 ± 0.4^{b}	7.0 ± 0.8^{c}	7.5 ± 1.0^{c}
S n-9	12.0	31.5 ± 0.7^{b}	26.7 ± 1.3^{c}	17.7 ± 0.9^{d}	$22.1 \pm 2.2^{c,d}$
S n-7	13.8	8.0 ± 0.3^{b}	9.8 ± 0.8^{c}	9.8 ± 0.3^{d}	$10.9 \pm 0.6^{c,d}$
S n-6	7.0	10.9 ± 0.5^{b}	11.2 ± 1.6^{b}	10.3 ± 0.8^{b}	10.6 ± 0.3^{b}
S n-3	31.5	0.7 ± 0.1^{b}	8.9 ± 1.0^{c}	16.1 ± 1.6^{d}	17.1 ± 1.7^{d}
SFA	33.8	48.5 ± 1.4^{b}	42.3 ± 1.2^{c}	$44.8 \pm 3.3^{b,c,d}$	37.6 ± 0.6^{d}
UFA	35.5	50.3 ± 1.4^{b}	47.2 ± 2.6^{b}	36.6 ± 1.5^{c}	$42.4 \pm 2.5^{b,c}$
HUFA	29.1	0.5 ± 0.1^{b}	8.2 ± 0.9^{c}	15.3 ± 1.2^{d}	15.2 ± 1.8^{d}

[a]Fatty acid composition in mol%. Means ± SEM of three experimental values. SFA, saturated fatty acids; UFA, 16- and 18-carbon unsaturated fatty acids; HUFA, 20- and 22-carbon highly unsaturated fatty acids; S n-9, sum of n-9 fatty acids; S n-7, sum of n-7 fatty acids; S n-6, sum of n-6 fatty acids; S n-3, sum of n-3 fatty acids; —, data lower than 0.5 mol%.
[b–d]Within a line values for brown adipose tissue that do not share the same superscript are significantly different at $P < 0.05$ or less.

(70:30:1, by vol) as the developing solvent. The stereospecific analysis of TAG was done on samples from three rats killed at the onset and after feeding periods of 3 d, 1 and 4 wk as described previously (2). Briefly, representative *sn*-1,2- and *sn*-2,3-acylglycerols were generated by Grignard degradation, and *rac*-phosphatidic acids were chemically synthesized. Fatty acids at the *sn*-2 position from *sn*-1,2-phosphatidic acids were released by phospholipase A_2. This allowed the direct determination of the fatty acids at positions 1 and 2 and the calculation of the fatty acids acylated at position 3. Fatty acids were determined directly following thin-layer chromatography by gas–liquid chromatography of the methyl esters (11) on a Chrompack 9000 chromatograph equipped with an AT-WAX capillary column (0.25 mm i.d. × 60 m; Alltech, Templeuve, France). All solvents contained 25 mg/L ethyl gallate as an antioxidant, and reactions were carried out under nitrogen.

Although reproducible results were obtained even when low levels of fatty acids were present, figures for minor fatty acids are not reported. Results are given as means ± SEM. The Student's *t*-test was used to establish significant differences among means.

RESULTS

TAG fatty acid composition. The fatty acid composition of TAG of BAT changed markedly during the first week of fish oil feeding. Thereafter (week 1 to week 4), no significant changes were observed. Based on these results, four time points were selected: time 0 (basal diet), after 3 d (about half the final steady level of HUFA concentration), and after 1 and 4 wk (final steady-state) (Table 1). There was a rapid and very significant increase (30-fold) in the concentration of HUFA in TAG of BAT, 88% of HUFA belonging to the n-3 series. After one week, HUFA reached a steady level corresponding to 15%, which was about half that in the fish oil diet. All individual n-3 HUFA reached a steady but different level after the same period. The final concentration of 22:6n-3 was significantly higher than that of 20:5n-3 (+60%, $P < 0.05$); thus the concentration of these HUFA was found in a proportion inverse to that in the fish oil diet (22:6n-3, 8.4%; 20:5n-3, 15.9%). The observed increase in HUFA was compensated for by a progressive decrease in UFA (mainly of the n-9 series; from 31.5 to 22.1% at 0 and 28 d, respectively; $P < 0.05$) and by a similar decrease in SFA (from 48.5 to 37.6% at 0 and 28 d, respectively; $P < 0.01$). Both UFA and SFA groups reached a steady level after 1 wk, as was also observed for HUFA.

Fatty acid distribution in TAG. The positional distribution of the main fatty acids in the BAT TAG of rats fed the basal and the fish oil diets is given in Table 2. In rats fed the basal diet, each position of TAG was characterized by a specific fatty acid pattern. Fatty acids at position 1 were highly saturated (about half of the total SFA were in this position), whereas positions 2 and 3 were mainly acylated with UFA (80% of the total amount). Only trace amounts of HUFA were found at each of the three positions. Among UFA, monoenoic acids of the n-7 series were evenly distributed, those of the n-9 series were distributed mainly at position 2 and 3, and 18:2n-6 was mainly acylated to

TABLE 2

Positional Distribution of Fatty Acids in Triacylglycerols of Brown Adipose Tissue from Rats Fed a Fish Oil Diet[a]

	0 d			3 d			1 wk			4 wk		
	Position			Position			Position			Position		
Fatty acid	1	2	3	1	2	3	1	2	3	1	2	3
14:0	1.5 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	1.3 ± 0.2	1.9 ± 0.0	1.5 ± 0.3	1.6 ± 0.1	2.9 ± 0.2	1.6 ± 0.4	2.1 ± 0.0	2.8 ± 0.1	0.8 ± 0.3
16:0	17.1 ± 0.5	6.6 ± 0.2	10.6 ± 0.7	16.5 ± 0.6	7.2 ± 6.1	6.1 ± 0.5	29.1 ± 1.4	4.6 ± 0.2	4.1 ± 0.8	14.2 ± 0.3	7.0 ± 0.3	3.9 ± 0.4
18:0	4.1 ± 0.2	1.6 ± 0.1	4.4 ± 0.3	3.4 ± 0.1	1.4 ± 0.1	2.5 ± 0.2	5.6 ± 0.4	0.7 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	1.0 ± 0.0	2.4 ± 0.3
18:1n-9	5.6 ± 0.2	13.3 ± 0.1	12.3 ± 0.5	4.7 ± 0.1	10.1 ± 0.6	11.2 ± 0.7	1.1 ± 0.1	9.5 ± 0.5	8.6 ± 0.6	3.4 ± 0.1	7.8 ± 0.1	9.2 ± 1.7
20:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	—	0.8 ± 0.1	0.6 ± 0.0	0.1 ± 0.0	0.6 ± 0.0
16:1n-7	1.7 ± 0.1	2.4 ± 0.1	2.0 ± 0.0	1.8 ± 0.2	2.9 ± 0.3	2.7 ± 0.1	1.0 ± 0.1	4.7 ± 0.1	1.6 ± 0.3	1.7 ± 0.1	3.2 ± 0.1	2.2 ± 0.4
18:1n-7	1.1 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	1.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	2.1 ± 0.2	0.9 ± 0.0	0.5 ± 0.0	2.2 ± 0.1	1.2 ± 0.0	0.5 ± 0.1
18:2n-6	1.3 ± 0.0	6.5 ± 0.5	2.6 ± 0.5	1.2 ± 0.2	6.4 ± 0.8	2.6 ± 0.5	0.9 ± 0.1	6.7 ± 0.3	1.1 ± 0.3	0.9 ± 0.0	5.9 ± 0.2	2.2 ± 0.2
18:4n-3	—	—	—	—	—	—	—	—	0.7 ± 0.0	—	—	0.7 ± 0.1
20:5n-3	—	—	—	0.7 ± 0.1	—	2.0 ± 0.2	1.3 ± 0.1	0.5 ± 0.0	2.9 ± 0.3	1.4 ± 0.1	0.5 ± 0.0	3.0 ± 0.3
22:5n-3	—	—	—	—	—	0.7 ± 0.1	—	—	1.4 ± 0.2	—	—	1.6 ± 0.2
22:6n-3	—	—	—	—	1.3 ± 0.2	1.7 ± 0.2	0.9 ± 0.1	2.4 ± 0.2	3.7 ± 0.6	1.3 ± 0.1	2.1 ± 0.4	4.8 ± 0.7
S n-9	5.7 ± 0.2	13.4 ± 0.1	12.5 ± 0.5	5.0 ± 0.1	10.2 ± 0.6	11.5 ± 0.7	3.0 ± 0.2	7.6 ± 0.5	7.6 ± 0.6	4.0 ± 0.1	7.9 ± 0.1	9.8 ± 1.7
S n-7	2.8 ± 0.1	3.0 ± 0.1	2.3 ± 0.1	4.3 ± 0.3	3.4 ± 0.3	3.2 ± 0.2	3.2 ± 0.3	4.2 ± 0.1	2.8 ± 0.4	3.9 ± 0.2	4.4 ± 0.1	2.7 ± 0.4
S n-6	1.5 ± 0.1	6.7 ± 0.5	2.9 ± 0.5	1.5 ± 0.2	6.6 ± 0.8	3.1 ± 0.5	1.2 ± 0.1	7.0 ± 0.3	2.0 ± 0.4	1.3 ± 0.1	6.3 ± 0.2	3.0 ± 0.2
S n-3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	1.6 ± 0.2	2.0 ± 0.3	5.2 ± 0.5	4.3 ± 0.6	5.4 ± 0.4	6.4 ± 0.6	3.7 ± 0.4	3.2 ± 0.6	10.2 ± 1.5
SFA	22.9 ± 0.5	9.7 ± 0.4	15.9 ± 0.7	21.3 ± 0.6	10.6 ± 0.3	10.4 ± 0.4	22.9 ± 0.9	10.8 ± 0.5	11.1 ± 1.6	19.9 ± 0.2	11.3 ± 0.4	7.2 ± 0.6
UFA	9.8 ± 0.4	23.1 ± 0.7	17.5 ± 1.1	10.4 ± 0.6	19.7 ± 1.6	17.1 ± 1.2	6.9 ± 0.5	18.4 ± 0.9	11.3 ± 1.3	8.9 ± 0.3	18.6 ± 0.3	14.9 ± 2.1
HUFA	—	—	—	1.5 ± 0.1	1.9 ± 0.2	5.1 ± 0.3	2.9 ± 0.2	3.3 ± 0.2	9.4 ± 0.6	3.5 ± 0.2	3.0 ± 0.4	10.1 ± 0.7

[a]Fatty acid composition in mole%. For each fatty acid or group of fatty acids the sum of the three positions equals its total amount in triacylglycerols. Means ± SEM of three experimental values. See legend to Figure 1 for other explanations and abbreviations.

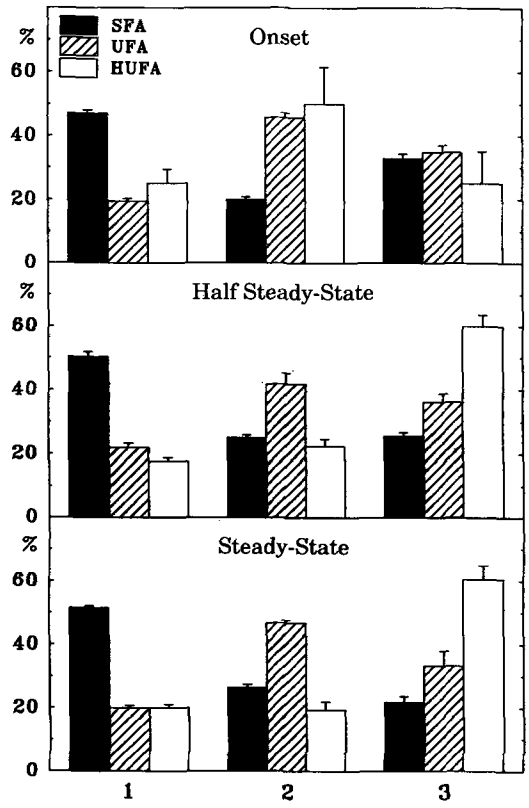

FIG. 1. Time-course of the changes in the positional distribution of the three fatty acyl groups in triacylglycerols of brown adipose tissue during fish oil feeding. Results are expressed in mole% for each series in each of the three position, the sum of the three positions being 100. Onset, basal diet; half steady-state, stage at which half changes in the 20- and 22-highly unsaturated fatty acids (HUFA) content were reached (day 3); Steady-state, mean value at the two last stages (weeks 1 and 4). Values are means (n = 3) and T-bars show SEM. SFA, saturated fatty acids; UFA, 16- and 18-unsaturated fatty acids.

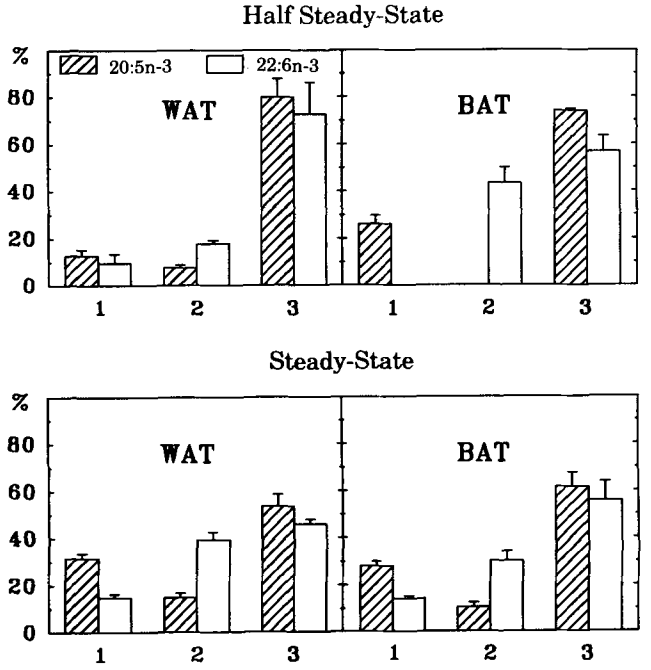

FIG. 2. Positional distribution of 20:5n-3 and 22:6n-3 in triacylglycerols of white (WAT) and brown (BAT) adipose tissues during fish oil feeding. Results are expressed in mole% for each series in each of the three positions, the sum of the three positions being 100. Half steady-state, day 7 for WAT and day 3 for BAT; steady-state, weeks 2–4 in WAT and weeks 1–4 in BAT. Data for WAT are computed from (1). Values are means (n = 3) and T-bars show SEM.

position 2 (about 60% of the total amount). In rats fed the fish oil diet, a significant decrease of the amount of both SFA and UFA at position 3 was observed after 3 d for the former ($P < 0.01$) and after 1 wk ($P < 0.05$) for the latter. The amount of UFA also decreased significantly at position 2 ($P < 0.01$), but this was not reflected in significant changes in the distribution of n-7 and n-9 fatty acids and of 18:2n-6. The rapid enrichment of HUFA in BAT was characterized by the rapid acylation of HUFA in position 3, where they mostly replaced SFA (after 3 d) and UFA (after 1 wk). To a lesser extent, HUFA were also acylated in positions 1 and 2, with the time courses of these changes being similar. The changes led to a 2.5-fold increase in the proportion of HUFA at position 3 ($P < 0.05$) at the expense of position 2, whereby the HUFA proportion at position 1 remained nearly steady (Fig. 1). When a half steady-state was reached, the distribution patterns of 20:5n-3 and 22:6n-3 were clearly distinct (Fig. 2). If one considers only relative values higher than 0.5 mol%, 22:6n-3 was found at positions 2 and 3 in roughly similar proportions, whereas 20:5n-3 was found at positions 1 and 3, its abundance being about three times lower at position 1 than at position 3 ($P < 0.01$). At the end of the feeding period (steady-state of HUFA content), about 60% of the total 20:5n-3 and 22:6n-3 were found acylated at position 3, with some 20:5n-3 present at position 2 (10% of the total), and with 22:6n-3 at position 1 (16%) (Fig. 2). The two other n-3 fatty acids, 18:4n-3 and 22:5n-3, were found only at position 3 (Table 2).

DISCUSSION

In the present study, the composition of the n-3 HUFA enriched diet differed greatly from that of BAT at the onset of fish-oil feeding (Table 1). The fatty acid supply through the intestine, liver and plasma was thus maintained at a high level that allowed the TAG pools to reach steady-states rapidly. Since a drastic dietary change was made, the data can primarily be interpreted in fatty acid metabolic terms. Thus, as suggested previously (12), the known selectivity of the acylating enzymes enabled us to distinguish the fatty acids between three primary groups (SFA, UFA and HUFA).

To our knowledge, this study is the first to examine TAG structure in BAT. In rats fed the basal diet, TAG of BAT contained almost exclusively SFA and UFA, and their distribution pattern appeared similar to that previously reported in WAT of the rat and other mammals (2,13–15). The preferential incorporation of SFA into position 1 and of 18:2n-6 into position 2 in BAT, as in WAT, is likely to result from a similar substrate affinity in the first two acy-

lating steps in phosphatidic acid biosynthesis. However, it is also possible that acyl-CoA concentrations could influence the site of fatty acid acylation in phosphatidic acid and help in overcoming the intrinsic specificities of acyltransferases (16,17).

Feeding of the fish oil diet induced different trends in fatty acid content and distribution in TAG of BAT (Figs. 1 and 2) in comparison with TAG of WAT (Fig. 2; Ref. 2). The SFA content progressively decreased in BAT, mainly at position 3, whereas it increased slightly in WAT without causing noticeable changes in distribution. The total amount of UFA decreased less in BAT than in WAT, but UFA positional distribution, including that of 18:2n-6, was unaffected in either fat depot. The HUFA enrichment in TAG was greater and more rapid in BAT than in WAT, but the distribution patterns of these exogenous fatty acids were similar.

Despite the increasing interest in the effect of n-3 fatty acids under various pathological conditions (6), the metabolic fate of the fatty acids involved has only been insufficiently investigated, especially with respect to their storage in and their mobilization from adipose tissues (18). Knowledge of the stereochemistry of stored TAG seems not only important with regard to the acylation steps but also with regard to the lipolysis processes. Indeed, the hormone-sensitive lipase from WAT is known to have a marked preference for the primary ester bond of TAG (19). This lipase was also shown to preferentially release polyunsaturated fatty acids from TAG in WAT (20). Similar positional specificity may be expected for the hormone-sensitive lipase of BAT, but fatty acid release cannot be assessed without knowledge of TAG structure. Noticeable differences are detected when the distribution of the two main n-3 HUFA in BAT (Fig. 2) is compared with that previously reported for TAG of WAT (2). At the steady-state, the higher enrichment of 20:5n-3 and 22:6n-3 in position 3 of TAG of BAT when compared to WAT suggests tissue-specific differences in stereospecificity at the level of the three acylating steps. This is corroborated by the observation of a delayed acylation of 20:5n-3 at position 2 and of 22:6n-3 at position 1. Unfortunately, in previous studies on these acylation processes in adipose tissue (21,22), the specificity for n-3 fatty acids was not considered. When comparing the time-dependent distribution of the two main n-3 fatty acids in TAG of BAT to that of WAT, some differences appear mainly at the level of 22:6n-3. In BAT, the proportion of this fatty acid decreased at position 2 and increased at position 1, whereas the proportion at position 3 was maintained. In WAT, the proportion of 22:6n-3 increased at positions 1 and 2 at the expense of position 3. This suggests that subtle mechanistic controls exist in the stepwise acylation of 22:6n-3 onto glycerol-3-phosphate. In particular, the early acylation of 22:6n-3 at position 2 in BAT suggests some tissue-dependent specificity of the 2-acylglycerophosphate acyltransferase. On the other hand, similarities between the two tissues were also observed. Thus, an early acylation of 20:5n-3 and 22:6n-3 at position 3 was found both in BAT and in WAT. This observation suggests that in both tissues the last step of TAG biosynthesis, controlled by the diacylglycerol acyltransferase, has a higher affinity for these fatty acids than that seen along the phosphatidic acid pathway. Molecular remodeling and differential acyltransferase specificities are likely responsible for the decreasing amount of 22:6n-3 at position 2 in BAT as well as for the delayed acylation in WAT of 20:5n-3 and of 22:6n-3 at positions 1 and 2, respectively. Important remodeling processes are likely to take place in BAT following the synthesis of phosphatidic acid since the enrichment of 22:6n-3 relative to 20:5n-3 in TAG was not observed in adipocyte choline or ethanolamine glycerophospholipids (Leray, C., Andriamampandry, M., Raclot, T., and Groscolas, R., unpublished data). The progressive changes that we observed in BAT fatty acid composition may furthermore result from selective hydrolysis of storage lipids and/or reesterification of n-3 HUFA taken up from blood. A similar hypothesis has been brought forward for WAT (23) and liver (24) TAG in fed and starved rats, respectively. The higher enrichment of BAT TAG in 22:6n-3 may involve a preferential release of 20:5n-3 over that of 22:6n-3, as has been suggested for WAT (18,25). The relative importance of these mechanisms in the build-up of fat depots during fish-oil feeding remains to be determined. Another explanation for the observed remodeling of TAG molecules in BAT leading to a difference in enrichment of 22:6n-3 relative to 20:5n-3 may be that the availability of specific fatty acids is altered by selective fatty acid oxidation. Since the dietary fat level is known to modulate the thermogenic activity of BAT (26–28), the high-fat diet used in this study is likely to have increased its mitochondrial oxidative capacity and peroxisomal metabolism. Although the importance of these two fatty acid degradation pathways has not yet been evaluated, a number of data suggests that they contribute efficiently to the oxidation of different fatty acids on the basis of their relative affinities (29). This is supported by observations of increased hepatic fatty acid oxidation in rats fed n-3 fatty acids (30–33) as well as observed differential oxidation of fatty acids (34). Thus, complex regulatory mechanisms are likely to be involved in promoting the synthesis of characteristic TAG molecular species in BAT when rats are fed a fish oil enriched diet. Conversely, it can be postulated that the fatty acids stored may modulate BAT thermogenic activity. Since the role of specific fatty acids as uncouplers for BAT adipocyte respiration remains unclear (9,27), future experiments with n-3 fatty acids are needed to advance our understanding of the regulation of this important physiological mechanism.

When comparing fatty acid metabolism in BAT and WAT in the course of dietary treatment, the relative weights of the two fat stores and their vascularization should also be considered. The rapid attainment of a steady-state fatty acid composition, as we observed here in BAT, may be due in part to its small size (about 0.25 g TAG vs. about 5 g in a rat retroperitoneal depot). Although the rate of *de novo* TAG formation in BAT was shown to be less than that in WAT (35), a high overall turnover rate of lipids in BAT could adequately explain the faster incorporation of n-3 HUFA into BAT compared to WAT.

In conclusion, it appears that BAT has a higher affinity than WAT for n-3 HUFA (2) when animals are fed a fish oil diet, although a contribution from intracellular fatty acid interconversion should not be ruled out. Our study of

the time-related acylation of n-3 HUFA in BAT shows specific patterns when compared to WAT. Our data suggest the existence of several regulatory steps (which may be sensitive to dietary manipulations) in the build-up of BAT fat depots. This process may include diet-induced changes in the thermogenic capacity of BAT. Further studies are required to determine whether the major function of this highly specialized tissue is affected by the stereochemistry of the TAG stored.

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Absorption of Isomeric, Palmitic Acid-Containing Triacylglycerols Resembling Human Milk Fat in the Adult Rat

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The effect of the positional distribution of palmitic acid (16:0) in triacylglycerols (TAG) on 16:0 apparent absorption in adult rats was investigated. The rats were fed two diets which contained 30 energy % as fat with identical total fatty acid compositions, both containing 30% 16:0. The Betapol diet contained TAG with 73% of total 16:0 in the *sn*-2 position, the control diet contained TAG with 6% of total 16:0 in the *sn*-2 position. After six weeks on these diets, the rats were killed two or six hours after the last meal, and the small intestine was removed, cut into 10-cm segments, and the fatty acid composition of the segment's contents was determined. At both time points the amount of 16:0 in the intestinal segments starting at 40 cm from the stomach was much lower in the animals fed Betapol than in the animals fed the control diet. Overall absorption of 16:0 and stearic acid was significantly greater in the Betapol group. Absorption of oleic and linoleic acid from the small intestine was similar in both groups, although the overall absorption was significantly greater in the animals fed Betapol. Total fat absorption was significantly higher in the Betapol-fed rats than in the control-fed rats. No effect on calcium and nitrogen absorption, on plasma total cholesterol and TAG levels, and on bodyweights (growth) was seen. The data demonstrate that the positional distribution of the fatty acids in the TAG molecule affects the site of absorption in the small intestine and particularly the net absorption of saturated fatty acids.

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The fat components of milk formulas used for bottle-feeding infants are usually based on vegetable oils which are either used alone or in combination with butterfat or other animal fats. The fat components are often formulated to approximate the total saturated fatty acid (FA) composition of human milk. Studies have demonstrated that human milk fat is better absorbed than infant formulas, particularly in the premature and newborn infant (1–7). Human milk fat typically contains 20–25% palmitic acid (16:0) (5), and most (>70%) of the 16:0 is esterified to the *sn*-2 position of the triacylglycerols (TAG). It has been suggested that this, together with the lipases present in human milk, accounts for its high absorption (8,9). In contrast, vegetable fat-based infant formulas containing similar 16:0 levels as human breast milk consist predominantly of *sn*-1,3 16:0 TAG, which are less readily absorbed than human breast milk even when residual bile salt-stimulated lipase activity is eliminated by sterilization (10). The apparent nutritionally beneficial effect of *sn*-2 16:0 TAG is thought to be related to the way saturated FA are absorbed by the intestine.

Little information is available on the apparent absorption of TAG with similar total FA composition but with different FA positional distribution. Previous studies on infants, as well as on laboratory animals, on TAG absorption have either compared animal vs. vegetable fats, fats with different FA compositions, or the effect of chemical randomization on fat absorption. The goal of the present study was to investigate the effect of FA positional distribution in TAG on TAG lipolysis and FA absorption. We have therefore prepared two structured fats with identical total FA composition but differing only in the positional distribution of 16:0, with the latter either being predominantly esterified to the *sn*-1,3 positions or the *sn*-2 position. The TAG were used to study the effect of FA positional distribution on TAG hydrolysis in the small intestine, the rate and extent of absorption of the lipid hydrolysis products, and the nature of the excreted products in the feces of adult rats.

MATERIALS AND METHODS

Chemicals. All solvents and chemicals (Merck, Darmstadt, Germany) used were at least analytical grade. Methyl heptadecanoate (99% pure) was obtained from Merck.

Diets. The composition of the 30 energy % semisynthetic diets is given in Table 1. The diets were prepared in one batch for the entire experimental period and were stored in small portions at −22°C. The animals were provided with demineralized drinking water starting from day five after their arrival.

TABLE 1

Composition of the Semisynthetic Diets

Ingredients	g/1000 kcal
Calcium caseinate	60.7
Vitamin mixture (Vit'70)[a]	1.0
Mineral mixture (RMH'88)[a]	3.6
Cellulose	15.0
Fat blend (control or test)	32.3
Maize starch	134.3
Total	246.9

[a]Amounts of minerals and vitamins in the purified diets per 1000 kcal: Ca, 846 mg; P, 728 mg; K, 601 mg; Na, 167 mg; Cl, 167 mg; Mg, 133 mg; Fe, 8.3 mg, I, 0.04 mg; Mn, 16.7 mg; Zn, 4.0 mg; Cu, 1.65 mg; choline chloride, 250 mg; myoinositol, 25 mg; vitamin B_{12}, 0.0129 mg; vitamin B_1 (thiamine mononitrate), 1.5 mg; calcium pantothenate, 5 mg; niacin, 5 mg; vitamin B_2 (riboflavin), 1.5 mg; vitamin B_6 (pyridoxin), 1.58 mg; folate (folic acid), 0.25 mg; vitamin K_3 (menadion, concentration 22.7%), 0.227 mg; biotin, 0.05 mg; vitamin A, 2500 IU; vitamin D_3, 250 IU; and vitamin E (concentration, 500 IU/g), 20 mg.

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Abbreviations: FA, fatty acids; FAME, fatty acid methyl esters; GC, gas chromatography; Ln, linoleic acid; O, oleic acid; S, saturated fatty acid; TAG, triacylglycerol; TC, total cholesterol; TLC, thin-layer chromatography.

TABLE 2

Fatty Acid (FA) Composition of the Fat Blends[a]

FA	Overall		*sn*-2 Position[b]	
	Control	Betapol	Control	Betapol
14:0	0.9	1.0	0.4	1.9
16:0	29.9	30.4	5.9	72.7
16:1	0.3	0.1	0.2	[c]
18:0	3.4	3.2	0.5	6.9
18:1n-9	50.0	51.5	68.2	14.7
18:2n-6	13.4	13.3	24.2	3.6
18:3n-3	0.3	0.1	0.4	[c]

[a]Percentages of individual FA are expressed relative to total FA methyl esters. The control blend was a mixture of natural oils (87.5% palmolein, 11% high oleic acid sunflowerseed oil, 1.5% sunflowerseed oil). Betapol was prepared by enzymic interesterification.
[b]To calculate the proportion of an FA esterified to the *sn*-2 position, the following formula applies: *sn*-2 position (%)/3 × overall FA methyl esters (%), e.g., for 16:0 in the test blend: 72.7/3 × 30.4 = 0.8 (80% of total 16:0 is esterified to the *sn*-2 position); or for 16:0 in the control blend: 5.9/3 × 29.9 = 0.066 (6.6% of total 16:0 is esterified to the *sn*-2 position).
[c]Less than 0.1%.

Fats. The *sn*-1,3 16:0 rich fraction was obtained by solvent fractionation of palm oil, followed by blending with 11% high-oleate sunflowerseed oil, and 1.5% sunflowerseed oil. The Betapol-rich fat was obtained by *sn*-1,3 enzymic interesterification of a palm fraction with sunflower FA, followed by solvent fractionation of the resultant TAG. Both fats were fully refined before use. FA and TAG compositions are shown in Tables 2 and 3, respectively.

Animals and feeding regimen. Forty male rats (Wistar WU; Charles River Wiga, Sulzfeld, Germany), seven weeks of age were housed individually in steel cages and had free access to food and drinking water. After an acclimatization period of two days, the animals were divided according to body weight into two groups of 20 animals each (Group 1 and Group 2). They were trained to eat their daily food in two separate meals, each during a 30 min time span, at 700 and 1900 hours. During this meal-type feeding period, the rats were randomly subdivided into two additional groups: half the animals of Group 1 were killed 2 h after the last morning meal (Group 1 or 2, 2 h) and the other half 6 h after the last morning meal (Group 1 or 2, 6 h). Each subgroup consisted of 10 animals. To ensure that the animals consumed the entire meal on the day of sacrifice, the animals were provided with a meal that was 90% of the usual amount.

TABLE 3

Silver-Phase Triacylglycerol (TAG) Analysis of Fat[a]

TAG[b]	Control	Betapol
SSS	0.3	0.3
SOS	12.0	0.9
OSS	2.8	6.6
SLnS	8.0	[c]
SSLn	1.4	3.1
SOO	43.9	5.8
OSO	1.0	54.1
OSLn	[c]	22.8
SOLn	13.6	1.8
OOO	12.6	0.8
>3 Double bonds/TAG	4.4	4.2
sn-2 Saturated FAME relative to total FAME	5.5	86.9

[a]Fat composition is given a weight percent relative to total TAG.
[b]S, saturated acid; O, oleic acid; Ln, linoleic acid; FAME, fatty acid methyl ester.
[c]Less than 0.2%.

Serum total cholesterol levels. A commercially available kit from Boehringer Mannheim (Boehringer Testcombination Cholesterol CHOD-PAP; Mannheim, Germany) was used to determine total cholesterol (TC). Cholesteryl esters in serum were hydrolyzed with cholesterol esterase to free cholesterol which, with cholesterol oxidase, formed hydrogen peroxide which was measured spectrophotometrically using a Vitatron Programmable Analyser PA 800 (Dieren, The Netherlands).

Serum TAG levels. Total glycerol was determined using the GPO-PAP method (Boehringer Mannheim). Free glycerol was determined enzymatically [using the Boehringer Mannheim ultraviolet (UV) method, omitting the KOH hydrolysis step]. TAG was defined as the difference between total glycerol and free glycerol.

Preparation of intestinal segments and collection of intestinal contents. The animals were decapitated either two or six hours after their last meal. The stomach was ligated just below the end of the oesophagus and just below the pylorus. The duodenum and ileum were ligated and cut into 10-cm pieces. The intestinal contents were collected as quickly as possible using blunt metal needles and placed into glass tubes containing cold methanol and Tris/HCl buffer (pH 4) (1:1, by vol). The mixtures were thoroughly agitated and placed on ice until further analysis within the same day. The entire intestinal content of each of the segments was thoroughly mixed with 4 mL cold methanol, immediately followed by addition of 1 mL of Michaelis buffer (containing sodium acetate and sodium barbital, pH 3) to stop any residual lipase activity. After mixing, 2 mL of chloroform and 50 µL of internal standard solution (methyl heptadecanoate, 17:0 methyl ester, 10 µg/µL) was added. After vortexing for 10 s, the mixture was left at room temperature for at least 15 min, and was then centrifuged for 10 min at 1500 × *g*. After centrifugation, the supernatant was collected, and the sediment was washed with 4 mL of chloroform/methanol (2:1, vol/vol), mixed well using a Vortex mixer, and again centrifuged for 10 min at 1500 × *g*. Two mL chloroform and 2 mL distilled water were added to the combined supernatants, and the mixture was cautiously shaken and subsequently centrifuged for another 10 min at 1500 × *g*. The chloroform layer was removed and washed with 8 mL methanol/water (1:1, vol/vol), carefully mixed, and centrifuged again. The extract was evaporated under a stream of nitrogen. Finally, 2 mL of chloroform were added, and the extract was stored under nitrogen at -20°C.

Preparation of fatty acid methyl esters (FAME) from the lipid extracts. An appropriate amount of lipid extract was concentrated under a stream of nitrogen in a screw-capped vial. After addition of 4 mL of methanolic HCl, the mixture was placed in a waterbath at 65°C for 2 h. After cooling to room temperature, 8 mL of 5% (wt/vol) aqueous

NaCl was added, and the reaction mixture was extracted with 6 mL of hexane/diethyl ether (1:1, vol/vol). The extraction was repeated twice with 3 mL of hexane/diethyl ether (1:1, vol/vol) under the same conditions, and the combined hexane/diethyl ether extracts were concentrated under a stream of nitrogen. The residue containing the FAME was purified by thin-layer chromatography (TLC) (DC Fertigplatten, Kieselgel 60; Merck) using hexane/diethyl ether (80:20 vol/vol) as developing solvent; detection was with 0.01% primuline under UV (254 nm) light. The zone corresponding to FAME was scraped off and extracted three times with hexane/diethyl ether (1:1, by vol/vol). After evaporation of the solvent, the residue was dissolved in 100 μL of *n*-heptane and transferred to gas chromatography (GC) vials and stored.

GC analysis. FAME were analyzed on a Varian 3700 chromatograph (Walnut Creek, CA) equipped with an automatic sampler and flame-ionization detector, employing a wall coated open tubular fused silica capillary column coated with 0.20 μm Chrompack (CP) Sil-88 (Fused Silica, Chrompack, Middelburg, The Netherlands) (length 50 m, i.d. 0.25 mm). The injector and detector temperature were set at 230°C. After an initial isothermal period of 8 min at 170°C, the temperature was raised to 220°C at 2°C/min, followed by a hold at 220°C for 10 min. Nitrogen was used as the carrier gas.

Silver-phase high-performance liquid chromatography. This method was used according to Jeffrey (11) to identify the specific TAG species present in the blend.

Feces collection and extraction of total fat in dried feces. Feces were collected and pooled from study days 20–27. The samples were stored under nitrogen at -70°C until further analysis. The freeze-dried feces (1.5–2 g) was placed into a 100-mL glass beaker. Five mL of 20% aqueous HCl was added per gram of feces and mixed. After 2 h, approximately 40 g of calcium sulfate was added, and the mixture was well stirred. The mixture was then quantitatively transferred to a Whatman thimble (Whatman, Maidstone, Kent, United Kingdom). The mixture was extracted with petroleum ether for 8 h, using a Soxhlet extractor (Whatman) and a preweighed 100 mL-flask. The petroleum ether was evaporated, and the extracted lipid was dried with acetone. The amount of extractable lipid was determined gravimetrically. After addition of a known amount of 17:0 as internal standard, the extracts were methylated and analyzed by GC.

Gross energy determination of feces (Parr adiabatic bomb). Gross energy in feces was determined by the International Standards Organization method (12).

Protein determination in feces using a Kjelfoss automatic instrument. The method used was that described by Smith (13).

Mineral determination in feces. Feces samples were dry-ashed, and the minerals were converted to their chlorides by fusing with concentrated HCl and then diluted with an equal volume of water. The metal chlorides were extracted with 3M aqueous HCl, and the resulting solutions were analyzed by atomic absorption spectroscopy or colorimetry.

Calcium determination in feces using atomic absorption spectroscopy. Calcium was determined according to the *AOAC Official Methods of Analysis* (14). The following formula was used to calculate the calcium content in the sample:

$$\text{Ca (wt\%)} = (c \times \text{dilution} \times 100)/(w \times 10^4) \quad [1]$$

in which c is the concentration in the diluted solution (μg/mL) and w the sample weight.

Statistical analysis. The Student's *t*-Test was used to analyze the rat feces data, food consumption and growth, utilizing the SAS statistical analysis package (version 6.4; SAS Institute, Cary, NC). The fatty acid analysis data of the intestinal segment contents were log-transformed prior to statistical analysis.

RESULTS

Fatty acid composition of the fat blends. Tables 2 and 3 show the composition of the fat blends used. The total FA compositions of the control and Betapol blends were identical. However, the blends substantially differed in the positional distribution of the FA on the TAG glycerol backbone. In the Betapol blend, 80% of 16:0 was esterified to the *sn*-2 position; in the control blend, it was only 6.6%. This affected the positional distribution of oleic acid—in the control blend oleic acid was predominantly esterified to the *sn*-2 position (44.6% of total), and in the Betapol it was only 9.5%.

The results of the silver-phase analysis (Table 3) show that in the control blend the major TAG were SOO (saturated–oleic–oleic) and SOLn (saturated–oleic–linoleic), as compared to the Betapol blend, in which OSO and OSLn predominated.

Bodyweight, growth and food consumption. The bodyweights at the start of the experiment of the different groups did not differ statistically and were as follows (g ± SEM): control (2 h), 201.6 ± 6.3 g; control (6 h), 202.8 ± 11.7 g; Betapol (2 h), 203.3 ± 6.5 g; Betapol (6 h), 199.4 ± 12.3 g. At the end of the experiment, the bodyweights also did not differ significantly and were: control (2 h), 266.7 ± 22.2 g; control (6 h), 255.5 ± 21.6 g; Betapol (2 h), 264.4 ± 15.4 g, Betapol (6 h), 261.4 ± 21.9 g.

No statistically significant differences between the rats fed the different diets in respect to food consumption or growth were seen. The mean total food intake (g ± SEM) over study days 20–26 was for the Betapol-fed animals (74.8 ± 1.25 g) and for the control-fed animals (70.5 ± 1.76 g). The mean growth (g ± SEM) from the start of the experiment up to study day 27 was for the Betapol-fed animals (65.6 ± 3.47 g) and the control-fed animals (58.9 ± 3.47 g).

Plasma TC and TAG levels of the rats at the end of the feeding period. Table 4 shows the plasma TC and TAG levels of the rats at the end of the feeding period, 2 and 6 h after the last meal. No significant differences were seen between the rats fed the different diets.

Rat feces analysis. Table 5 shows the overall fat and FA absorption during the feeding period of the different diets. The rats on the Betapol diet absorbed significantly more fat than those on the control diet—97.2% vs. 95.6% ($P < 0.001$), respectively. The mean FA absorption of the rats

TABLE 4

Total Plasma Cholesterol (TC) and Triacylglycerol (TAG) Levels of Rats[a]

	TC (mM)		TAG (mM)	
Group	2 h	6 h	2 h	6 h
Control	1.78 ± 0.5	1.79 ± 0.3	2.3 ± 0.5	2.14 ± 0.8
Betapol	1.84 ± 0.4	1.88 ± 0.3	2.48 ± 0.8	2.92 ± 1.1

[a]At the end of the feeding period 2 and 6 h after the last meal (n = 10, mean ± SD).

fed the Betapol diet was significantly higher (99.2%) compared to that of the control-fed rats (97.9%, $P < 0.001$). The increased fat absorption also resulted in a small but significant increase in the total energy absorption from the Betapol diet and, consequently, in an increase in digestible energy.

No statistically significant differences were seen in respect to nitrogen and calcium excretion.

In Table 5 data are shown on the individual FA excreted in the feces during a 5-day period. Palmitic acid, oleic acid, linoleic acid and stearic acid were all significantly better absorbed by the rats fed the Betapol blend than by the rats fed the control blend. The greatest difference in excretion between the two diets was found for 16:0.

Fatty acid analysis of the intestinal segment contents. Figure 1 shows the results of the FA analyses of the different intestinal segments contents. It appears that, in particular 16:0, when fed as Betapol, required a much smaller portion of the intestine to be absorbed when compared to the control blend; this was seen both at 2 and 6 h

TABLE 5

Rat Feces Analysis

Parameter	Control	Betapol
Fat intake (g/d/rat)	1.97 ± 0.23	2.09 ± 0.23
Fat absorption (%)	95.6 ± 0.5	97.2 ± 0.5[a]
Fatty acid absorption (%)	97.9 ± 0.5	99.2 ± 0.3[a]
Energy absorption (%)	92.93 ± 1.2	93.65 ± 0.37[b]
Digestible energy (MJ/kg diet)	18.64 ± 0.24	18.79 ± 0.07[b]
Calcium absorption (%)	63.33 ± 5.49	63.98 ± 6.17
Nitrogen absorption (%)	95.79 ± 0.93	95.71 ± 0.58
Total lipid excreted (mg/d/rat)	86 ± 16	58 ± 10[a]
Total lipids excreted consisted of		
Fatty acids (mg/d/rat)	41 ± 12.3	16.3 ± 6.0[a]
% of intake	2.08 ± 0.55	0.78 ± 0.28[a]
16:0 (mg/d/rat)	21.2 ± 8.4	3.4 ± 1.2[a]
% of intake	3.28 ± 1.16	0.51 ± 0.18[a]
18:0 (mg/d/rat)	5.14 ± 1.46	2.94 ± 1.1[a]
% of intake	6.4 ± 1.7	3.5 ± 1.2[a]
18:1 (mg/d/rat)	4.4 ± 1.42	1.12 ± 0.54[a]
% of intake	0.45 ± 0.13	0.12 ± 0.05[a]
18:2 (mg/d/rat)	0.58 ± 0.15	0.24 ± 0.11[a]
% of intake	0.35 ± 0.08	0.14 ± 0.06[a]

[a,b]Statistically significantly different from control blend (*t*-test) at [a]$P < 0.001$ or [b]$P < 0.01$.

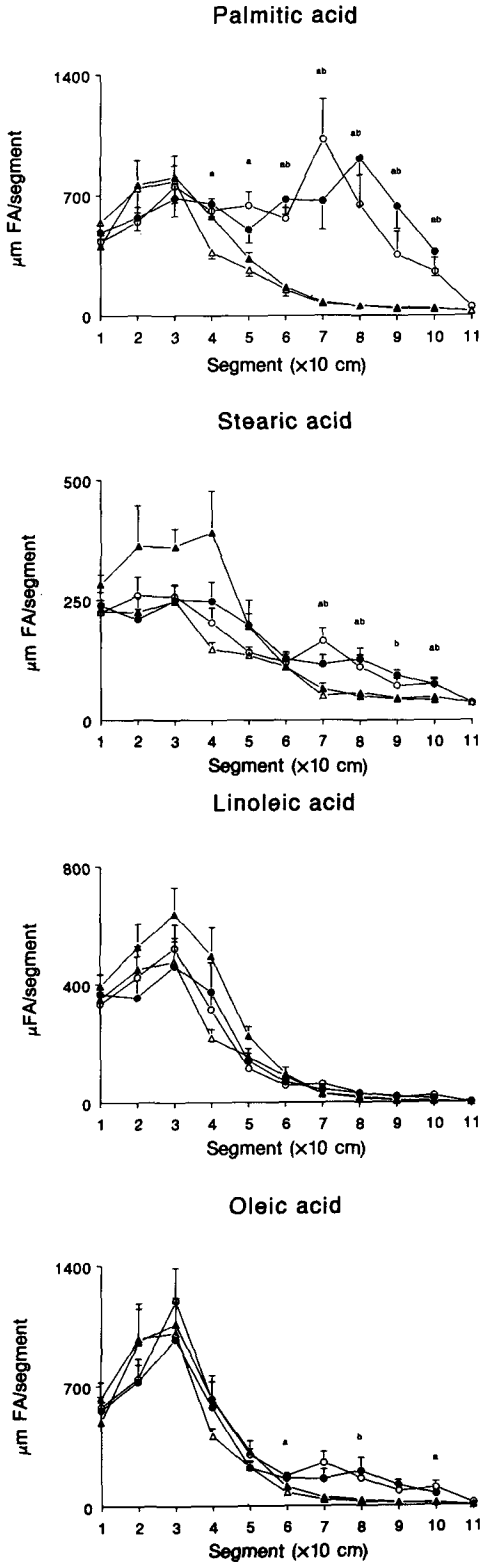

FIG. 1. Nonabsorbed fatty acids, palmitic, stearic and linoleic and oleic (µg/segment ± SEM) in intestinal segments. The data were log-transformed before statistical analysis. Level of significance, $P < 0.05$. Open symbols, 2 h after the last meal; closed symbols, 6 h after the last meal. Triangles, Betapol blend, circles, control blend; n = 6 rats per group. a, Betapol differs significantly from control at two hours after the last meal; b, Betapol differs significantly from control at six hours after the last meal. FA, fatty acid.

after the last meal. Furthermore, oleic acid was significantly better absorbed from segment 6 onward by the rats fed Betapol compared to the animals fed the control diet both at 2 and 6 h after the last meal; the differences, however, are small. The same holds true for the absorption of stearic acid. From segment 6 onward, stearic acid present in the Betapol blend was better absorbed than stearic acid present in the control blend. No explanation can be given for the observed increase in stearic acid in the first four segments at 6 h after the last meal of the control blend compared to the other blend and other time points. The absorption patterns for linoleic acid and oleic acid did not differ between the two diets.

DISCUSSION

In the present study, we investigated the effect of the positional distribution of FA in dietary TAG on the rate of apparent digestion and absorption in the rat. The two fat blends used had identical FA compositions but differed in the positional distribution of 16:0 and oleic acid. In the control blend, most of the total 16:0 (93.4%) was esterified to the *sn*-1,3 positions of the TAG, whereas in the test blend (Betapol) 16:0 was predominantly esterified to the *sn*-2 position of TAG.

The total fat excretion of the rats was significantly lower on the Betapol diet than on the control diet. This is mainly due to the decreased excretion of 16:0 on the Betapol blend. Tomarelli *et al.* (4) found that the relative absorption of 16:0 was linearly related to its proportion at the *sn*-2 position of the TAG molecule, which agrees with our results.

Furthermore, our data clearly demonstrate that both at 2 and 6 h after the last meal, 16:0 fed to the rats as Betapol appeared to be more rapidly absorbed in the upper part of the intestine as compared to 16:0 of the control diet. This more rapid disappearance of 16:0 was related to a more efficient absorption overall. For 18:1, preferentially esterified to the *sn*-2 position in the control blend as compared to the Betapol blend, no differences in the absorption patterns between control and Betapol blend were seen. The absorption of the other FA (stearic and linoleic acid) required about the same intestinal length for both fat blends. These results indicate that the positional distribution of saturated FA in dietary TAG is more critical in determining absorption efficiency than it is for unsaturated FA.

The differences in intestinal length required to absorb 16:0 located at the *sn*-2 vs. the *sn*-1,3 positions can be explained as follows. First, TAG containing unsaturated FA at the *sn*-1,3 positions are more readily hydrolyzed than TAG bearing saturated FA at these positions, and thus they are absorbed earlier (15–17). Second, after TAG hydrolysis, the products released are for the Betapol blend oleic acid and *sn*-2-palmitoylglycerol, and for the control blend 16:0, oleic acid and *sn*-2-oleoylglycerol. The absorption of unsaturated monoacylglycerol is strongly favored due to its relatively greater hydrophilicity as compared to saturated monoacylglycerols (18). Third, 16:0 (melting point, 63°C) is not as well absorbed as are fatty acids with a lower melting point, and it requires more bile acids to maintain it in micellar solution, which is a prerequisite for lipid absorption to occur. Finally, FA soaps can be formed, especially in the absence of a micellar phase. Calcium soaps have been shown to be about six times less soluble in the intestinal lumen than are the corresponding free FA (19). Calcium and magnesium soaps of unsaturated FA are more soluble in the intestinal fluid and can be solubilized to a greater extent by the products of fat digestion and thus are better absorbed (20,21). Furthermore, calcium soaps are in general much more soluble in unsaturated FA micelles than in saturated FA micelles.

To get some insights on the amount of FA soaps and free FA present in the feces, an attempt was made to differentiate between the two forms by solvent-extracting the feces samples before and after acidification. It was found that 70–75% of the total FA present could be extracted only after acidification of the sample (data not shown). This suggests that the bulk of the FA excreted in the feces was present as FA soaps. That this did not result in significantly altered calcium excretion is perhaps not surprising, because of the very low levels of fat being excreted in comparison to fecal calcium. Of the latter, as little as 20% may have been associated with FA as soaps (assuming a 2:1 molar ratio for FA/calcium), making it very difficult to detect changes in calcium associated with this pool. Mattson and co-workers (20) reported that the excretion of stearic acid in feces was affected by the amount of calcium present in the diet and by the positional distribution of stearic acid in the TAG. In their experiments they used 6 mg calcium per g of diet, whereas our diets contained 3.4 mg/g diet.

Our results do not suggest that differences in FA positional distribution lead to differences in plasma TC and TAG levels in the rat. Several papers have been published on the possible effect of FA positional distribution in the TAG molecule on cholesterol metabolism in rabbits and in humans (22,23). It has been reported by Ockner and coworkers (24) and Ockner and Jones (25) that differences in intestinal absorption could account for the physiological characteristics of chylomicrons in rats. In the light of our findings, it is thus possible that the positional distribution of FA in the TAG could affect some aspects of chylomicron metabolism. Zampelas *et al.* (26) have studied the effect of a liquid meal containing either the Betapol blend or the control blend on postprandial lipid responses. They reported that the positional distribution of palmitic acid on TAG did not effect the postprandial lipemia in adult male subjects. However, their results do not exclude the possibility that different responses may be seen when these TAG are given for a longer period of time.

In conclusion it is clear that feeding TAG enriched in *sn*-2 16:0 (Betapol) to rats as compared to *sn*-1,3 16:0 TAG results in increased total fat absorption. In addition, the length of intestine required to achieve this absorption is reduced. These results were obtained on adult rats having a well developed intestinal system. In weanling animals, and in premature or newborn infants, the uptake systems are not as well developed (lower levels of lipases and bile acids), and it appears reasonable to hypothesize that under these conditions the *sn*-2 16:0 fat blend will have even greater beneficial effects (27,28).

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n-3 Fatty Acids Inhibit Defects and Fatty Acid Changes Caused by Phenytoin in Early Gestation in Mice

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Our previous work has shown that n-3 fatty acids exert a protective effect against phenytoin-induced cleft palate when phenytoin was administered midgestation [gestational days (GD) 12 and 13] to CD-1 mice. The effects of dietary n-3 fatty acids on phenytoin teratogenicity were investigated at an earlier gestational period (GD 9) to examine whether n-3 fatty acids could exert protective action against other teratogenic effects of phenytoin apart from cleft palate. The effect of phenytoin exposure on maternal hepatic polyunsaturated fatty acid composition was also studied since Δ6 desaturase activity has been shown to be modified by pharmacological action. Female CD-1 mice were fed a standard laboratory diet (SLD), safflower oil (SAFF) or a cod liver/linseed oil (CLO/LO)-based diet for three weeks prior to impregnation and throughout pregnancy. Pregnant mice were administered a single i.p. dose of phenytoin on GD 9, and teratological assessments were performed on GD 19. Tissues were harvested on GD 10 for maternal hepatic phospholipid fatty acid analysis from another group of phenytoin-treated mice. The CLO/LO and the SLD mice, as compared to the SAFF-fed animals, showed a reduction in total malformations and fetal growth retardation due to phenytoin. Open eye defect was the only anomaly induced by phenytoin in the CLO/LO fetuses while phenytoin produced a variety of malformations in the SAFF fetuses such as tail defects, cleft palate, open eye and absence or blockage of the ureter. Dietary n-6/n-3 fatty acid ratios were reflected in maternal hepatic phospholipids; however, phenytoin exposure appeared to inhibit the conversion of 18:2n-6 to 20:4n-6 in the SAFF dams only. These results indicate that the overall protective effects of dietary n-3 fatty acids on phenytoin embryo-toxicity are observed at an early gestational period.

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Phenytoin (5,5-diphenylhydantoin, Dilantin), an extensively used anticonvulsant, is a suspected human teratogen (1–3) and is teratogenic in mice (4–9). The Fetal Hydantoin Syndrome (FHS) describes a particular set of anomalies in children born to women taking hydantoin anticonvulsants during pregnancy, primarily phenytoin (1). This syndrome of defects includes craniofacial anomalies, cardiac defects, and growth and mental retardation (1). Mouse models have been used frequently to investigate issues related to the teratogenic actions of phenytoin. Chronic *in utero* phenytoin exposure resulting in a mouse hydantoin syndrome similar to FHS has been reported (9). In various mouse strains, acute phenytoin administration during organogenesis results primarily in the induction of cleft palate, cardiac defects, open eyelid and fetal weight reduction (4–9). As with other classic teratogenic agents, the type of defect produced by acute phenytoin administration depends upon the stage during pregnancy when phenytoin exposure occurs.

A number of possible mechanisms of phenytoin teratogenicity have been suggested including: (i) drug-induced folate deficiency; (ii) a glucocorticoid-mediated mechanism; and (iii) metabolism to a reactive intermediate and binding to embryonic macromolecules (10). The mechanism of phenytoin teratogenicity under investigation in our laboratory is the prostaglandin H synthase (PGS)-mediated bioactivation of phenytoin to a reactive intermediate (11). Indication of a teratogenic role for reactive intermediates of phenytoin has come from *in vivo* studies showing that covalent binding of phenytoin to embryonic proteins was positively and highly correlated with phenytoin-induced malformations (12). As PGS occurs in high concentrations in embryonic tissues (13), it may play an important role in the metabolic activation of phenytoin to a reactive intermediate. Evidence for a teratogenic role for a reactive free radical intermediate generated from PGS-mediated metabolism of phenytoin includes studies demonstrating that: (i) inhibition of PGS activity by administration of acetylsalicylic acid in CD-1 mice resulted in a 50% reduction in phenytoin-induced cleft palates and a 43% reduction in covalent binding of phenytoin to embryonic proteins (14); (ii) addition of either antioxidants or the PGS inhibitor, indomethacin, inhibited arachidonic acid (20:4n-6)-dependent covalent binding of phenytoin to microsomal proteins from ram seminal vesicles (a rich source of PGS) and various murine tissues (11); and (iii) pretreatment with either the antioxidant caffeic acid or the free radical spin trapping agent, α-phenyl-*N*-*t*-butyl-nitrone, caused dose-related decreases in phenytoin-induced cleft palates and resorptions (14).

The present study represents a follow-up to our previously published report (12) which demonstrated that a semipurified diet rich in n-3 fatty acids fed for three weeks prior to gestation was associated with a lowered frequency of cleft palate due to phenytoin exposure on gestational days (GD) 12 and 13 in CD-1 mice. Additionally, on GD 12, embryonic production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and covalent binding of radiolabeled phenytoin to embryonic proteins were reduced, and the embryonic n-3/n-6 phospholipid fatty acid ratio was increased in animals fed the n-3 fatty acid-enriched diet. The results of these studies led to the hypothesis that an underlying mechanism of the protective effects of the n-3 fatty acids was an inhibition of embryonic PGS-mediated bioactivation of phenytoin. Since phenytoin-induced cleft palate was the sole defect

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; CLO/LO, cod liver oil/linseed oil; FAME, fatty acid methyl esters; FHS, Fetal Hydantoin Syndrome; GD, gestational day; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGS, prostaglandin H synthase; PUFA, polyunsaturated fatty acids; SAFF, safflower oil; SLD, standard laboratory diet; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

observed following phenytoin treatment on GD 12 and 13, it was unclear whether the protective effects of n-3 fatty acids were primarily mediated against phenytoin-induced cleft palate or whether n-3 fatty acids could exert protective action against a variety of teratogenic effects of phenytoin. Before any specific conclusions can be drawn, it is necessary to determine if the protective effect of n-3 fatty acids against phenytoin-induced malformations in the CD-1 mouse exists on a continuum from an earlier period of organogenesis (GD 9) to the time of palatal shelve closure on GD 12 and 13. Administration of phenytoin on GD 9 was chosen in the present study since phenytoin administration in mice during this period of organogenesis has been associated with an increased incidence of open eyelid and tail defects (15).

Animals lack the enzymes necessary to form n-3 or n-6 polyunsaturated fatty acids (PUFA) and thus require these essential fatty acids in the diet. Microsomal fractions from the brain, and other animal tissues are capable of elongation and desaturation of the n-3 and n-6 essential fatty acids. Two of the endproducts of the elongation and desaturation pathway, eicosapentaenoic acid (20:5n-3) and 20:4n-6, each generate a separate family of prostaglandins *via* PGS metabolism. Of major significance for essential fatty acid nutrition is the competitive desaturation of the n-3 and n-6 series by Δ6 desaturase as this is the rate-limiting step of the elongation–desaturation pathway (16). As a consequence, supplementation of diets with rich n-3 sources, such as fish oils, results in the selective incorporation of 20:5n-3 and docosahexaenoic acid (22:6n-3) into various lipid classes while concomitantly reducing the content of 20:4n-6 in several tissues as has been seen in humans and experimental animals (12, 17,18). The increased tissue n-3 fatty acid content can directly inhibit PGS activity by causing a decreased peroxide tone (19). Additionally, recent studies have shown that tissue PUFA content and composition may be profoundly altered by pharmacological modification through inhibition of Δ6 desaturase with possible functional implications (20). As eicosanoids derived from n-3 or n-6 PUFA in maternal and fetal tissues play a vital role in fetal growth (13), research is needed to determine whether phenytoin treatment alters tissue PUFA levels during pregnancy and whether this is affected by the family of PUFA concurrently consumed.

In the present study we have extended our previous investigation (12) and have addressed these important issues. A set of reproductive endpoints as well as maternal phospholipid fatty acid profiles were determined so that the impact of dietary n-3 fatty acids on the relationship of phenytoin exposure with (i) reproductive toxicity during an early period of pregnancy (GD 9), and (ii) PUFA metabolism could be studied at the level of the individual animal.

MATERIALS AND METHODS

Animals and mating procedure. Virgin female (20–25 g) CD-1 mice (Charles River Canada, St. Constant, Quebec, Canada) were housed in a temperature-controlled room (24 ± 1°C) with a 12-h light–dark cycle (light cycle 0700 to 1900 h). The mice were housed in polypropylene shoebox cages with chrome-plated wire lids and heat-treated hardwood bedding (Beta-Chip; Northeastern Products Corp., Warrensburg, NY). They were randomly housed four per cage, and food and tap water were provided *ad libitum*. For mating purposes, one to three females from the same cage were housed with a male from 1700 to 0830 h. Food was removed from the cages during this period. The presence of a vaginal plug the following morning confirmed insemination and this date was designated as GD 1. Females were returned to their dietary treatment cages where they remained until sacrifice.

Diets. The semipurified diets used in these experiments were modified versions of the AIN-76 diet (21) consisting of the following ingredients (g/kg diet): dextrose, 489; vitamin-free casein, 182; D,L-methionine, 3; cellulose, 51; vitamin mix, 12; mineral mix, 63; lipid, 200. The lipid component was either 200 g safflower oil (SAFF) or a mixture of 20 g SAFF, 90 g codliver oil (CLO) and 90 g linseed oil (LO). The 20 g SAFF in the CLO/LO diet was included to provide a sufficient amount of linoleic acid (18:2n-6) to prevent essential fatty acid deficiency (21). All dietary components were obtained from ICN Biomedicals (Cleveland, OH). Vitamin and mineral mixes formulated for the increased requirements of these compounds during pregnancy were adapted from Fergusson and Koski (22). As CLO contained additional amounts of vitamins A and D, retinyl palmitate (102150 IU/kg diet) and cholecalciferol (9000 IU/kg diet) were added to the SAFF diet to match the vitamin concentrations in the CLO/LO diet. Additional *dl*-α-tocopheryl acetate was added to increase the overall concentration of vitamin E activity to 2000 IU/kg diet to protect the mice from an increased risk of *in vivo* lipid peroxidation and vitamin E insufficiency demonstrated previously in mice fed elevated concentrations of fish oil (23,24). The synthetic antioxidant butylated hydroxytoluene (BHT; ICN Biomedicals) was added to the dietary oils (0.01%, w/w) to protect against oxidation of PUFA. A standard laboratory diet (SLD; Mouse Chow® No. 5015; Purina Mills Inc., St. Louis. MO) was used as a control diet. The fatty acid composition of the diets is described in Table 1.

Semipurified diets were mixed before the beginning of each experimental period, aliquoted and stored under N_2 at -20°C. Aliquots contained sufficient food for approximately three weeks and were stored at 4°C under N_2. Female mice consuming semipurified diet were provided fresh diet on alternate days for three weeks prior to mating and throughout pregnancy. This feeding protocol has previously been shown to increase the ratio of n-3 to n-6 fatty acids in embryonic and maternal tissue phospholipids in mice fed 10% (w/w) of CLO/LO (1:1, w/w) (12). Food consumption and body weight data were recorded three times per week for the semipurified diet groups.

Samples of uneaten semipurified diet were removed from feed containers after each 48 h feeding period and stored at -20°C, under N_2 until analysis for thiobarbituric acid reactive substances (TBARS) to obtain an index of lipid peroxidation. A modification of the thiobarbituric acid (TBA) assay by Dahle *et al.* (25) was used. The lipids of each of the diet samples were extracted with chloro-

TABLE 1

Fatty Acid Composition of SLD, SAFF and CLO/LO Dietary Fats[a]

FAME[c]	Diet[b] SLD	SAFF	CLO/LO
14:0	1.44	1.72	3.08
16:0	24.44	5.64	10.55
16:1n-7	2.45	0.10	2.85
18:0	8.50	2.24	3.10
18:1n-9	34.79	73.12	26.15
18:2n-6	25.25	16.44	11.93
20:4n-6	0.10	0.17	1.06
18:3n-3	2.18	0.34	30.85
18:4n-3	nd[d]	nd	1.08
20:5n-3	nd	nd	4.88
22:5n-3	nd	nd	0.46
11:6n-3	nd	nd	4.01
UI[e]	95.5	108	205
n-6/n-3	11.70	48.85	0.31

[a]% (w/w) of total fatty acid methyl esters (FAME) = (weight of an individual FAME/weight sum of all FAME identified for a sample) multiplied by 100.
[b]SLD, standard laboratory diet; SAFF, safflower oil; CLO/LO, cod liver oil/linseed oil.
[c]FAME are identified by the number of carbon atoms and the number of double bonds, followed by the position of the first double bond from the methyl end "n-" of the fatty acid.
[d]Not detectable, less than 0.004% (w/w).
[e]UI (unsaturation index) = Σ (% FAME × number of double bonds in the FAME).

form/methanol (2:1, vol/vol) by the method of Folch *et al.* (26). Chloroform extracts were evaporated to dryness under N_2 and reconstituted with 40% ethanol to a final concentration of 0.5 mg/mL. To the reaction mixture was added 2.0 mL of 0.1 N sodium phosphate buffer (pH 6.0), 1.0 mL of 20% trichloroacetic acid and 2.0 mL of a 0.67% aqueous solution of TBA. After incubation for 15 min at 100°C, TBARS were measured spectrophotometrically at 535 nm on a Beckman DU-40 spectrophotometer. Analysis of the uneaten semipurified diet samples for TBARS yielded values that are indicative of relatively low levels of dietary lipid peroxidation (23,27), i.e., below 20 nmol of reference standard (malonaldehyde *bis*diethyl acetal); Sigma Chemical Co., St. Louis, MO) per g diet.

Experiment 1. A teratogenic dose of phenytoin (65 mg/kg) (5,12) was given to five SLD dams and ten mice each in the SAFF and CLO/LO groups as a single intraperitoneal (i.p.) injection on GD 9, employing a standard injection volume of 10.0 mL/kg body weight. A single dose regimen of phenytoin was given to decrease the likelihood of maternal toxicity or excessive embryo lethality which could mask the teratogenic effects of phenytoin (28). An equivalent number of dams in each dietary group received an identical volume of pH-matched vehicle (saline). Phenytoin (sodium diphenylhydantoin; Sigma) was dissolved in normal saline containing 0.002 M NaOH (Malinckrodt, Paris, KY) to achieve a final pH of 10.5. All solutions were prepared immediately prior to administration. Dams were killed on GD 19 by cervical dislocation. Following laparotomy, the uterus was exteriorized and the number, location and weight of fetuses and resorptions noted. All fetuses were weighed, sexed and examined for the presence or absence of major external anomalies (e.g., exencephaly, spina bifida, hydrocephaly, eye defects, hairlip and limb defects). Fetuses that died during a 2 h observation period after caesarian section were distinguished from live fetuses and resorptions in order to serve as an indication of postpartum mortality. Fetuses were also examined under a dissecting microscope for cleft palates. The frequency of malformations or of a specific defect was calculated as the number of affected fetuses divided by the total number of fetuses examined for a particular group and then multiplied by 100. The resorption incidence was determined as the number of resorptions divided by the number of implantation sites (fetuses plus resorptions) and expressed as a percentage.

Experiment 2. To examine the effects of dietary and phenytoin treatment on maternal hepatic phospholipid fatty acid composition, a second study was done in which phenytoin was administered to four mice from each dietary regime on GD 9 as described above. An additional four mice from each dietary group were injected with pH-matched vehicle. Maternal livers were extracted between 18 and 24 h after phenytoin or vehicle exposure on GD 9, rinsed with ice-cold 1.15% KCl and frozen at -80°C until phospholipid fatty acid analysis.

Tissue phospholipid fatty acid analysis. Lipid extracts from maternal liver homogenates were prepared as described previously (12) and separated into neutral lipid and phospholipid fatty acid fractions according to the method of Juaneda and Rocquelin (29). As described previously (12), fatty acid methyl esters (FAME) of total phospholipids were prepared and gas–liquid chromatography was used to separate and quantify FAME. FAME were identified by comparing retention times with those of authentic standards (Nu-Chek-Prep, Elysian, MN) and were quantified relative to heptadecanoic acid (17:0) used as internal standard.

Statistical analyses. Analyses of continuous variables were done as a three-by-two factorial design for two-way analysis of variance (ANOVA). The effects of diet and GD 9 phenytoin treatment on mean fetal weight were determined by nested ANOVA. As there were differences among the treatments in terms of litter size, statistical comparisons on fetal weight were performed using litter size as a covariate. When significant differences occurred, treatment mean differences were identified by Tukey's (equal cell sizes) or Bonferroni's (unequal cell sizes) multiple comparison tests. Statistical evaluations of postpartum mortality, the frequency of anomalies and specific types of malformations on a per-fetus basis were done by chi-square with Yates correction. All analyses were performed using the Statistical Analysis System for personal computers (Version 6.04, 1992; SAS Institute, Cary, NC). A probability of $P < 0.05$ was accepted *a priori* as the minimal level of significance for all analyses.

RESULTS

Maternal indices. Average feed consumption per cage of mice, either before or during pregnancy, was unaffected

by the type of semipurified diet (data not shown). Greater weight gain observed in the SLD mice during pregnancy as compared to the SAFF and CLO/LO dams may partially be explained by the larger litter size and the greater number of implantation sites of the SLD dams (Table 2). Litter sizes, expressed as the average number of live fetuses per litter, for the phenytoin-treated and control CLO/LO groups were lower than those for the SLD groups, whereas only the SAFF control dams had a smaller mean litter size relative to the SLD groups. Although the mean litter size of the phenytoin-treated SAFF diet group was larger than that of the control SAFF group, this may be partially explained by the greater number of implantation sites in the phenytoin-treated SAFF group compared to its control group. Comparison of implantation sites per dam gave the same results as those obtained for litter size with the additional observation that the mean number of implantation sites was greater in the phenytoin-exposed SAFF group vs. the phenytoin-exposed and control CLO/LO groups. Despite the differences between groups in terms of the average number of live fetuses and implantations per litter, neither drug nor dietary treatment affected the incidence of resorptions (Table 2). This result supports the idea that differences detected between the phenytoin-exposed SAFF group and both of the CLO/LO groups in terms of implantation sites were not likely due to treatment effects.

Fetal indices. Phenytoin exposure reduced mean fetal weight only in SAFF fetuses, whereas the fetuses from the n-3 fatty acid supplemented mice and the SLD group were unaffected by phenytoin treatment (Table 3). Although comparisons between the diet and drug groups in terms of postpartum mortality were not statistically significant, a clear trend was observed for an increased postpartum mortality due to phenytoin exposure in each of the dietary treatments (Table 3). When the phenytoin-treated groups were pooled, however, phenytoin was observed to exert a significant effect upon postpartum lethality relative to the groups receiving dietary treatment alone. Cleft palate, a defect characteristically induced by exposure to phenytoin on GD 11 through 13, was present following phenytoin treatment in SAFF fetuses but absent in fetuses from the SLD and CLO/LO groups (Table 3). In contrast to the presence of cleft palates observed in SAFF fetuses, the frequency of open eye defect was increased significantly by phenytoin treatment only in the CLO/LO diet group (Table 3). Although open eye defect was observed in phenytoin-exposed SAFF fetuses (2.6%), those fetuses affected were within one litter, whereas fetuses with open eye defect were distributed among four litters in the CLO/LO diet group.

Phenytoin exposure on GD 9 increased the frequency of malformations in each diet group (Table 3), although this increase was not statistically significant in the SLD group. The frequency of phenytoin-induced malformations was 2.5 times higher in SAFF offspring (16.2 %) as compared to CLO/LO fetuses exposed to phenytoin (6.4%). Malformations observed in the SAFF fetuses exposed to phenytoin on GD 9 included tail defects (8.5%), cleft palate (4.3%), open eye (2.6%) and absence or blockage of the ureter (1.7%). In contrast, open eye defect was the only malformation exhibited by the CLO/LO phenytoin-exposed fetuses. Anomalies observed in SLD offspring exposed to phenytoin included open eye (3.9%), tail defects (2.6 %) and one fetus with an atrophied hind region. The incidence of abnormalities did not differ significantly among the groups receiving dietary treatments alone and was within the norm for CD-1 mice fed either standard laboratory diets (5) or semipurifed diets (12).

Maternal hepatic phospholipid fatty acids. Dietary fat composition did not affect feed intake, prior to mating or during pregnancy, nor did it affect net weight gain in mice used for this analysis (data not shown). Phenytoin treatment on GD 9 decreased weight gain within each diet group, although the decrease was only significant within the CLO/LO group. The decrease in weight gain following

TABLE 2

Effects of Dietary Fat and Phenytoin Treatment on Pregnancy Outcome in Mice[a,b]

Treatment		Maternal	Live fetuses	Implantations	Resorption
Diet	Drug	weight gain (g)	per litter	per litter	incidence[c] (%)
SLD	Control	(5)[d] 32.20 ± 3.49[e]	13.20 ± 1.16[e]	14.00 ± 1.18[e]	6 ± 2
	Phenytoin	(5) 28.84 ± 1.03[e,f]	12.67 ± 0.33[e]	13.50 ± 0.62[e]	6 ± 2
SAFF	Control	(10) 21.99 ± 0.84[f,g]	9.70 ± 0.60[g]	10.50 ± 0.60[f]	7 ± 3
	Phenytoin	(10) 22.04 ± 1.31[f,g]	11.70 ± 0.45[e,f]	12.80 ± 0.49[e]	8 ± 2
CLO/LO	Control	(10) 20.47 ± 1.29[g]	10.40 ± 0.65[f,g]	11.10 ± 0.57[f]	7 ± 2
	Phenytoin	(10) 20.54 ± 1.59[g]	9.40 ± 0.98[f,g]	10.30 ± 1.08[f]	8 ± 1

[a]CD-1 mice were fed SLD, SAFF or CLO/LO based diets for 3 wk prior to implantation until gestational day (GD) 19. Dams were injected i.p. with either 65 mg/kg phenytoin or saline (control) on GD 9. Dams were caesarian sectioned on GD 19 and teratology assessment performed. See Table 1 for other abbreviations.

[b]All values except for the number of dams per treatment are expressed as the mean ± SEM.

[c][(number of resorptions)/(number of implantation sites) per dam] multiplied by 100.

[d]Number in parentheses is equal to the number of dams.

[e-g]Means not sharing a common superscript, within a column, are significantly different ($P < 0.05$) by Bonferroni's multiple comparisons test.

TABLE 3

Effects of Dietary Fat and Phenytoin Treatment on Fetal Indices[a]

Treatment		Fetal	Postpartum	Cleft	Open	Total
Diet	Drug	weight (g)	mortality	palates	eyes	malformed
SLD	Control	(66)[b] 1.37 ± 0.02[c,d]	2 (3.0%)[f]	0 (0%)	1 (1.5%)	1 (1.5%)
	Phenytoin	(76) 1.28 ± 0.01[d,e]	6 (7.9%)	0 (0%)	3 (3.9%)	6 (7.9%)
SAFF	Control	(97) 1.41 ± 0.01[d]	1 (1.0%)	2 (2.1%)	0 (%)	2 (2.1%)
	Phenytoin	(117) 1.23 ± 0.01[e]	6 (5.1%)	5 (4.3%)	3 (2.6%)	19 (16.2%)[g]
CLO/LO	Control	(104) 1.30 ± 0.01[d,e]	3 (2.9%)	0 (0%)	0 (0%)	0 (0%)
	Phenytoin	(94) 1.29 ± 0.01[d,e]	5 (4.8%)	0 (0%)	6 (6.4%)[h]	6 (6.4%)[h]

[a]Mice were treated as previously described in footnote a of Table 2; SLD, standard laboratory diet; SAFF, safflower oil; CLO/LO, cod liver oil/linseed oil. See Table 1 for abbreviations.
[b]Number of fetuses examined.
[c]Mean ± SEM.
[d–e]Means not sharing a common superscript, within a column, are significantly different ($P < 0.05$) by Bonferroni's multiple comparisons test.
[f]Number of fetuses affected (% of fetuses affected within the treatment group).
[g]Significantly greater than SAFF control group ($P < 0.01$) and phenytoin-treated CLO/LO group ($P < 0.05$) by continuity-adjusted chi-square analyses.
[h]Significantly greater than CLO/LO control group ($P < 0.05$) by continuity-adjusted chi-square analysis.

phenytoin exposure could be due to a sedative-like effect of the drug. There were no effects of diet or drug exposure on the average number of live embryos or on implantation sites per litter (data not shown). In contrast to mice in Experiment 1, phenytoin caused a significant increase in the incidence of resorptions in the CLO/LO animals. This increase may have been due to an unusually low number of resorptions in the CLO/LO control group.

Individual fatty acid levels (% wt of total FAME per group) of the maternal liver phospholipid fraction are shown in Figure 1. The livers of control SLD and CLO/LO dams had a higher content of 16:0 and lower concentrations of 18:0 and 18:1n-9 as compared to SAFF control livers. The hepatic phospholipid concentrations of 18:2n-6 were the highest in the vehicle-control SLD group while the CLO/LO dams receiving vehicle showed higher hepatic phospholipid levels of 18:2n-6 than the SAFF control livers. The SAFF livers of control dams contained the highest amount of 20:4n-6, more than fourfold higher concentrations than were observed in control CLO/LO livers. The livers of SLD mice receiving saline vehicle also had higher levels of 20:4n-6 than did the CLO/LO control livers. The CLO/LO liver phospholipids contained the only detectable concentrations of 20:5n-3. The longer chain 22:6n-3 was seen in all dietary treatments but was dramatically affected by dietary treatment as CLO/LO livers contained more than five times and almost twice the concentrations in SAFF and SLD livers, respectively.

Phenytoin exposure also induced differences in the fatty acid composition of maternal liver phospholipids. Phenytoin treatment increased 18:2n-6 concentrations in SAFF and SLD maternal hepatic phospholipids. In contrast, phenytoin exposure did not affect hepatic phospholipid fatty acid composition in the CLO/LO group. The ratio of hepatic phospholipid 20:4n-6 to 18:2n-6 (Fig. 2) in livers from the SAFF dams was decreased as a result of phenytoin exposure, suggesting an inhibition in the conversion

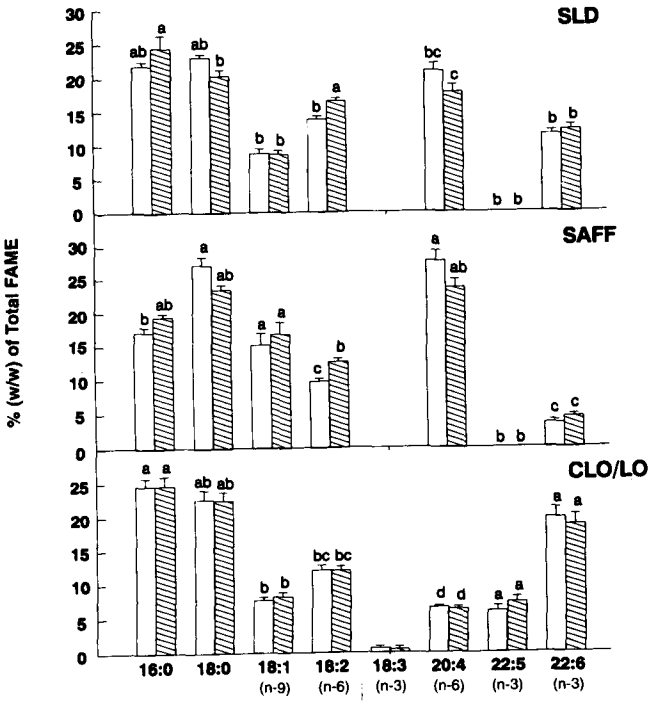

FIG. 1. Maternal hepatic phospholipid fatty acid composition in pregnant mice. Dams were fed either standard laboratory diet (SLD), safflower oil (SAFF) or codliver oil/linseed oil (CLO/LO) based diet for 3 wk prior to mating and through gestational day (GD) 10. On GD 9 dams were injected i.p. with saline (control) or with 65 mg/kg phenytoin. Dams were killed 24 h later and maternal livers were dissected, rinsed with ice-cold 1.15% KCl, and frozen at −80°C until fatty acid analysis. Data are expressed as the mean ± SEM (n = 4). Open bars represent vehicle controls and cross-hatched bars represent phenytoin-treated dams. Differences in the mean amounts for a particular fatty acid between diets and drug treatment are indicated by different letters as determined by Tukey's multiple comparisons test ($P < 0.05$). FAME, fatty acid methyl esters.

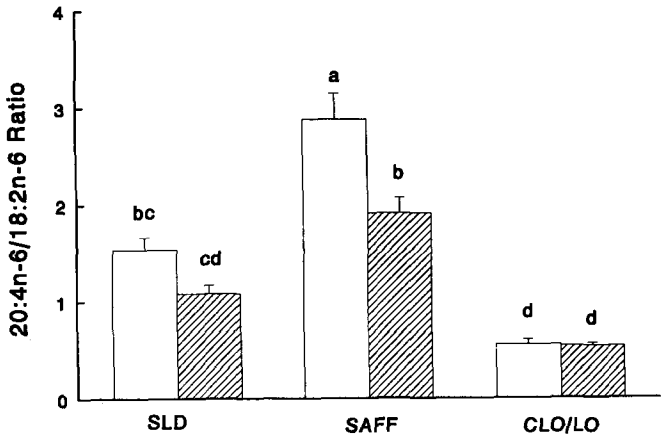

FIG. 2. Ratio of 20:4n-6/18:2n-6 in hepatic phospholipid fatty acids in pregnant mice. Mice were treated as described in Figure 1. Data are expressed as the mean ± SEM (n = 4). Open bars represent vehicle-controls and cross-hatched bars phenytoin-treated dams. Different letters indicate significant differences between means among all treatment groups by Tukey's multiple comparisons test ($P < 0.05$). See Figure 1 for abbreviations.

of 18:2n-6 to 20:4n-6 by phenytoin. Phenytoin treatment also decreased the ratio of n-6:n-3 hepatic phospholipid fatty acids in the SAFF group (Table 4) whereas the SLD and CLO/LO groups were unaffected by phenytoin in this regard. As expected, the CLO/LO groups exhibited the lowest phospholipid fatty acid n-6:n-3 ratio, and the n-6/n-3 ratio for the SLD mice was between those for the other two diet groups. Dams fed the SAFF diet had the lowest unsaturation index, likely due to the low amount of 22:6n-3 in the hepatic phospholipids (Table 4). The highest unsaturation index for hepatic phospholipid fatty acids came from those mice fed the CLO/LO diet while mice from the SLD groups yielded intermediary values.

DISCUSSION

As expected, acute phenytoin exposure on GD 9 in CD-1 mice produced adverse effects on fetal growth and morphological development that were similar to those previously observed in *in vivo* murine studies (5–9,12). The effects were evidenced by a decrease in fetal weight and an increased incidence of cleft palate, open eyelid and tail defects. Although phenytoin increased the frequency of malformations in CLO/LO fetuses (6.4%), there were 2.5 times more phenytoin-induced defects observed in fetuses from the SAFF group (16.2%). The lowered incidence of phenytoin-induced anomalies in the CLO/LO fetuses as compared to SAFF fetuses demonstrates that dietary n-3 fatty acids can decrease teratogenicity caused by phenytoin exposure at an early gestational period (GD 9), and these findings parallel earlier results (12) obtained on n-3 fatty acid-fed mice exposed to phenytoin with midgestation. Since resorption incidence was not affected by dietary treatment, the CLO/LO dietary treatment caused a decrease in phenytoin teratogenicity rather than a repartitioning of the type of outcome. Although phenytoin treatment was associated with an increase in anomalies in both of the semipurified diet groups and the SLD group (7.9%), the increase in SLD mice was not statistically significant. This latter finding was likely due to the relatively smaller number of dams in the SLD groups. The reduction in fetal weights observed in the SAFF diet group due to phenytoin exposure early during gestation is in agreement with other reports of phenytoin-mediated growth reduction (5–9,12,30). The phenytoin-mediated fetal growth retardation was suppressed in the CLO/LO group demonstrating that dietary n-3 fatty acid supplementation can also protect against this aspect of phenytoin fetotoxicity. This latter finding, however, contrasts our earlier work (12) which showed no protective action of n-3 fatty acids against fetal growth retardation induced by phenytoin. The lack of protection of the CLO/LO diet in our previous study could be related to the later gestational times (GD 12 and 13) of phenytoin administration. The earlier period of phenytoin treatment in the present study is also likely responsible for the relatively low incidence of phenytoin-induced cleft palate among the treatment groups, and this has also been observed in mice treated with phenytoin on GD 9 in other studies (15). Previous work by Lum and Wells (5) has indicated that the period of maximal sensitivity to phenytoin-induced cleft palate in the CD-1 mouse occurs on GD 12 and 13. On the other hand, in this study the total malformation incidence in the phenytoin-treated safflower oil group was relatively high at 16% and is similar to the total malformation incidences of approximately 10% seen in previous studies using CD-1 mice following phenytoin treatment on GD 12 and 13 (5,14).

TABLE 4

Unsaturation Index and n-6/n-3 Ratio of Maternal Hepatic Phospholipids (wt% of total FAME)[a]

	SLD		SAFF		CLO/LO	
	Control	Phenytoin	Control	Phenytoin	Control	Phenytoin
UI[b]	189 ± 4.95[c,d,e]	185.6 ± 6.83[d,e]	165.5 ± 5.93[e]	162.5 ± 4.85[e]	208.0 ± 8.86[d]	206.9 ± 9.69[d]
n-6/n-3	3.06 ± 0.16[f]	2.84 ± 0.15[f]	10.87 ± 0.94[d]	8.23 ± 0.38[e]	0.71 ± 0.06[g]	0.70 ± 0.04[g]

[a]CD-1 dams were fed either SLD or a SAFF or CLO/LO based semipurified diet, from 3 wk prior to implantation until gestational day (GD) 10. Dams were injected i.p. with either 65 mg/kg phenytoin or saline (control) on GD 9. Dams were killed on GD 10 and livers were immediately dissected, rinsed with ice-cold 1.15% KCl and frozen at -80°C until phospholipid fatty acid analysis. See Tables 1 and 2 for abbreviations.
[b]UI = Σ (% FAME × number of double bonds in the FAME).
[c]Means ± SEM.
[d-g]Means not sharing a common superscript, within a row, are significantly different ($P < 0.05$) by Tukey's multiple comparison test.

Dietary fat also affected the type of defects observed following phenytoin exposure on GD 9. Malformations observed in fetuses from the phenytoin-treated SAFF dams affected the tail, palate, eyes and the external opening of the urogenital system. Open eye defect, on the other hand, was the only gross defect induced by phenytoin in the CLO/LO group. Acute phenytoin exposure by single injections on GD 9 through 15 has shown that treatment on GD 9 alone leads to phenytoin-induced open eye defect in Swiss Webster mice (31). Phenytoin may affect the production of a precursor involved in eyelid formation and/or eyelid fusion since eyelid growth does not occur until GD 13–15 (32). Formation of the peridermal cells of the developing eyelid region begins on about GD 10 (33), and phenytoin treatment on GD 9 may interfere with the formation of this group of cells which are important in temporary fusions, such as those between the eyelids of a neonatal mouse (34).

The overall protection of n-3 fatty acids against phenytoin-induced growth retardation and the decreased incidence of overall malformations in the CLO/LO fetuses supports the idea that n-3 fatty acids exert a generalized protective action against phenytoin teratogenicity. The mechanisms involved could include an enhanced balance of bioactivation to detoxification of phenytoin in the SAFF-fed animals at the level of the embryo as our previous findings showed enhanced concentrations of covalently bound phenytoin to protein in embryos from SAFF-fed dams relative to CLO/LO embryos (12). This effect was attributed to the relatively higher level of embryonic activity of prostaglandin H synthase in SAFF embryos which can bioactivate phenytoin to a reactive intermediate. Differences among the dietary treatments in terms of distribution, conjugation and excretion pathways of phenytoin could also alter the plasma halflife and embryonic exposure to phenytoin, thereby modifying teratogenic outcome.

It is unlikely that the high intake of n-6 fatty acids contributed to the increased teratogenic action of phenytoin. Our earlier work (12) showed that mice fed either a saturated fat diet or a safflower oil-based diet demonstrated a similar incidence of phenytoin-induced cleft palate that was relatively higher in comparison to mice fed a CLO/LO diet. Moreover, studies have consistently shown that increasing the intake of 18:2n-6 above approximately 1–2% of energy does not lead to a further increase in 20:4n-6 tissue levels (35). Our previous findings showed no increase in embryonic, placental or hepatic levels of 20:4n-6 with 12% (w/w) safflower oil intake vs. 10% (w/w) hydrogenated coconut oil + 2% (w/w) safflower oil (12). These observations support our contention that the n-3 fatty acid-based diets exerted a protective effect on phenytoin teratogenicity as opposed to an enhancement of phenytoin teratogenicity by the n-6 fatty acid-based SAFF diet.

In our study, the CLO/LO diet produced changes in hepatic phospholipids consistent with the expected effect of CLO/LO, namely an increase in 20:5n-3 and 22:6n-3 contents and a reduction in 20:4n-6. This is in agreement with other reports (17–19) which indicate that the decrease in 20:4n-6 is the result of the competitive replacement of n-6 PUFA by n-3 PUFA and/or the inhibition of the Δ6 desaturase and elongase enzymes. Differences in the dietary ratio of n-6/n-3 between the SAFF (49) and CLO/LO (0.3) diets also resulted in a 15-fold reduction in this ratio for hepatic phospholipids from dams fed the CLO/LO diet relative to SAFF dams.

The present study provides new information on the effects of phenytoin on fatty acid composition during gestation. Phenytoin exposure increased 18:2n-6 concentrations in liver phospholipids from the SLD and SAFF mice but not in the CLO/LO hepatic phospholipids. The conversion of 18:2n-6 to 20:4n-6 was also reduced in the SAFF group exposed to phenytoin as compared to the SAFF control group as determined by calculating the ratio of product (20:4n-6) to precursor fatty acid (18:2n-6). This result is similar to the findings of Cunnane *et al.* (36), who found that phenytoin treatment of children with epidermolysis bullosa dramatically increased 18:2n-6 while simultaneously decreasing 20:4n-6 in plasma and erythrocyte phospholipids. These findings implied that Δ6 and/or Δ5 desaturation of 18:2n-6 was inhibited by phenytoin. Other studies had shown that microsomal Δ5 and Δ6 desaturase activity can be decreased upon exposure to xenobiotics. For example, hepatocarcinogenic treatment in rats, in the form of diethylnitrosamine and 2-acetylaminofluorene, was shown to result in a similar decreased 20:4n-6/18:2n-6 ratio in the hepatic phospholipids (20). In the present study, the possible inhibitory effect of phenytoin on the 18:2n-6 to 20:4n-6 conversion was not observed in hepatic phospholipids from the SLD or CLO/LO dams. This may have been due to the higher degree of affinity of the desaturases for the n-3 fatty acids (16). The higher amounts of n-3 fatty acids (i.e., lower ratios of n-6/n-3 fatty acids) in the hepatic phospholipids of the SLD and CLO/LO groups as compared to the SAFF dams, in conjunction with the higher affinity of the desaturase enzymes for the n-3 fatty acids, could have contributed to the lack of effect of phenytoin on the conversion of 18:2n-6 to 20:4n-6 observed in these groups. This idea is supported by the fact that 18:3n-3 conversion to 20:5n-3 and 22:6n-3 was not affected in the SAFF hepatic phospholipids.

In summary, the results from this study have shown that animals receiving a diet in the form of CLO/LO had a lower incidence of malformations as compared to SAFF-fed mice when treated with phenytoin in early gestation. Although further study is required, the protective effects of the CLO/LO diets could be attributed to its lower n-6/n-3 fatty acid ratio. The present findings, together with our previous work (12), indicate that semipurified diets containing n-3 fatty acids can strongly protect against a variety of malformations induced by phenytoin during two substantially different developmental periods of susceptibility to major phenytoin-induced defects. The PUFA composition of maternal liver phospholipids can be manipulated *in vivo* by administration of phenytoin and by administration of specific dietary oils. The present study revealed that the incorporation of long-chain n-3 PUFA into tissue phospholipids appears to influence the efficacy of phenytoin in depressing maternal liver phospholipid PUFA composition. Further work is needed, however, to fully evaluate the effect of linseed oil and fish oil feeding on the mechanisms of the phenytoin-induced alterations of hepatic PUFA metabolism.

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Effects of Low Casein and Fish Oil on Hyperlipidemia and Proteinuria in Nephritic Rats

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The effects of amino acid-fortified low casein and fish oil (FO) diets on hyperlipidemia and proteinuria were studied in rats with nephrotoxic serum nephritis. After an antiserum injection, rats were maintained for 14 d on four different experimental diets: a 20% casein diet containing corn oil (CO) or FO, or an 8% casein diet supplemented with cystine plus threonine containing CO or FO. The 8% casein diets reduced urinary protein excretion in nephritic rats without inducing severe growth retardation or fatty liver compared with the basal 20% casein diets. Both the 8% casein diet and the FO diet decreased serum cholesterol, triglyceride and phospholipid levels in nephritic rats, and nonesterified fatty acid levels were decreased by FO feeding. In nephritic animals, hepatic cholesterol synthesis was decreased by the 8% casein diets compared with the 20% casein diets, and tended to be reduced by FO feeding between groups at the same casein levels. No effect of diet was observed on fatty acid synthesis among the nephritic rats. FO administration to the nephritic animals suppressed fecal steroid excretion. While lipoprotein lipase activity was unchanged among the nephritic rats, hepatic triglyceride lipase activity was reduced by either the 8% casein or FO diet. The results suggest that the hypolipidemic action of low casein diets may, at least in part, be due to reduced hepatic cholesterol synthesis and suppressed triglyceride secretion from the liver. They also suggest that the hypolipidemic action of FO may, at least in part, be due to reduced hepatic cholesterol synthesis and decreased fatty acid mobilization from peripheral adipose tissue.

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Renal injuries such as puromycin aminonucleoside nephrotic syndrome (1) and nephrotoxic serum nephritis (NSN) (2) are known to be accompanied by severe hyperlipidemia. These types of hyperlipidemias need to be remedied as they are believed to cause further deterioration of kidney function (3,4).

Brenner *et al.* (5) suggested that injury of the glomerulus is caused by increased permeability of the glomerular capillary to plasma proteins and that high protein intake enhances the hyperfiltration of glomeruli causing progressive damage. It has been reported that low protein diets ameliorate proteinuria in nephrotic patients (6) and in rats (7). Protein malnutrition can occur after a long period of low protein intake, even if low protein diets have beneficial effects on symptoms of renal diseases. We demonstrated that low protein diets suppressed hyperlipidemia and proteinuria incident to NSN (8) and that the supplementation of limiting amino acids to diets low in proteins alleviated growth retardation due to low protein intake (9).

Dietary fish oil (FO) rich in n-3 polyunsaturated fatty acids has been shown to bring about greater hypolipidemic effects in rats through reduced hepatic lipogenesis (10) than corn oil (CO) which is rich in linoleate (18:2n-6). FO-enriched diets have also been reported to weaken the progression of autoimmune diseases in NZB/NZW (11) and MRL-1pr (12) mice by altering several immunological functions (13). The effects of FO diets on nephrotoxic serum nephritis, which is one of the immune-mediated models, remain ambivalent. There have been reports that FO alleviated this type of nephritis compared to beef tallow (14), while a high α-linolenate diet has been shown to exacerbate it when compared to a high linoleate diet (15).

In the present study, we examined the effects of dietary FO and of an amino acid-fortified low casein diet, individually and in combination, on the incidence of hyperlipidemia and proteinuria in NSN.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (three weeks old) were obtained from Charles River Japan (Kanagawa, Japan). The rats were kept on a stock pellet diet (CE-2; CLEA Japan, Tokyo, Japan) for 3 d. On the fourth day of preliminary feeding, rats were divided into two groups with equal body weights and were fed a basal 20% casein diet containing either 10% CO or FO for another three weeks. The rats were kept in an air-conditioned room at a temperature of 22 ± 2°C and at a relative humidity of 60 ± 5% using an 8:00 a.m. to 8:00 p.m. light cycle. All the animals were moved on the twenty-first day of preliminary feeding from individual cages with wire bottoms into metabolism cages to collect urine. The composition of the basal 20% casein diet was as follows: 20% casein (Oriental Yeast Co., Tokyo, Japan), 10% CO (Hayashi Chemicals Co., Tokyo, Japan) or FO (Sanomega EPA-S18GA, the generous gift of NOF Corporation, Tokyo, Japan), 63.3% α-corn starch (Nihon Nosan Kogyo Co., Yokohama, 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 composition of CO and FO is shown in Table 1. The FO contained 0.3% vitamin E. Separately from the vitamin mixture, the CO diet was therefore supplemented with extra vitamin E (Riken E Oil 800, Riken Vitamin Co., Tokyo, Japan) to adjust to the vitamin E content (350 mg/kg diet) of the FO diet. No side effects have been noted at this dose of vitamin E in rats (16). On the twenty-fourth day of preliminary feeding

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Abbreviations: 8-C, an 8% casein diet, supplemented with cystine and threonine, containing corn oil; 20-C, a 20% casein diet containing corn oil; 8-F, an 8% casein diet, supplemented with cystine and threonine, containing fish oil; 20-F, a 20% casein diet containing fish oil; BA, bile acids; Ch, cholesterol; CO, corn oil; FA, fatty acid; FO, fish oil; GBM, glomerular basement membrane; HTGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; NEFA, nonesterified fatty acids; NS, neutral sterols; NSN, nephrotoxic serum nephritis; PL, phospholipid; TG, triglyceride; TX, thromboxane.

TABLE 1

Fatty Acid Composition of Corn (CO) and Fish Oils (FO) (%)

Fatty acid	CO	FO
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
20:5n-3	trace	17.7
22:6n-3	trace	11.8

(day 0), rats of the CO and FO groups were further divided into two groups of equal body weight; they received a single intravenous injection in the tail vein of anti-rat kidney glomerular basement membrane (GBM) rabbit antiserum (0.5 mL/rat), which was produced by immunizing rabbits with the supernatant of trypsin-digested rat GBM (17,18). On the following day (day 1), the animals were subcutaneously immunized with rabbit γ-globulin (8 mg/rat; Sigma Chemical Co., St. Louis, MO) in 0.2 mL of Freund's complete adjuvant (Wako Pure Chemical Industries) in the hind foot pads as described previously (19,20). Immediately after the injection of antiserum (day 0), rats of each group were kept for 14 d on a particular experimental diet. Rats previously maintained on the CO diet were successively fed either a 20% casein diet containing CO (20-C) or an 8% casein diet supplemented with 0.3% L-cystine (Nippon Rikagakuyakuhin Co., Tokyo, Japan) and 0.36% L-threonine (Ajinomoto Co., Inc., Tokyo, Japan) containing CO (8-C), while those on the FO diet were fed either a 20% casein diet containing FO (20-F) or an 8% casein diet supplemented with 0.3% L-cystine and 0.36% L-threonine containing FO (8-F). Fats given no anti-serum injection were regarded as the normal group and maintained on the 20-C diet throughout the experiment. Each experimental diet was adjusted to 100% by changing the amount of α-corn starch to keep all the diets isocaloric. Water and all diets were available *ad libitum*. Urine excreted during the preceding 24 h by the rats individually housed in metabolism cages was collected at 9:00 a.m. each day. The animals were deprived of their diets at 9:00 a.m. on day 14, but allowed free access to water until sacrifice by decapitation 4 h later. Blood was collected and left to clot at room temperature to obtain serum. Liver, kidney and epididymal adipose tissue were quickly removed, washed with cold 0.9% NaCl, blotted on filter paper, and weighed. Aliquots of the liver and adipose tissue were frozen in liquid nitrogen and stored at -80°C until analyzed.

Lipid analyses. From the liver, total lipids were extracted according to the procedure of Folch *et al.* (21). After aliquots of the chloroform phase had been dried, cholesterol (Ch) (22), triglyceride (TG) (23) and phospholipid (PL) (24) levels were determined as described previously (8). The serum TG and PL levels were also determined as described above. Serum total Ch levels were enzymatically determined using a commercial kit (Wako Pure Chemical Industries). Serum nonesterified fatty acid (NEFA) levels were also determined as described previously (25).

Serum albumin and urinary protein determination. Serum albumin was determined with a commercial kit (Wako Pure Chemical Industries). Urinary protein was measured by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA) (26).

Cholesterol and fatty acid syntheses from [^{14}C]acetate in liver slices. Liver slices weighing 100–120 mg were incubated for 2 h in 1 mL of Krebs-Ringer phosphate buffer (pH 7.4) containing 37 kBq/μmol of [1-^{14}C]acetate (2.04 MBq/μmol; Amersham International plc, Buckinghamshire, United Kingdom). Total fatty acid (FA) and Ch syntheses were estimated as described previously (27,28).

Fecal steroid excretion. Feces were collected individually for 2 d before rats were sacrificed. Neutral sterols (NS) and bile acids (BA) were extracted according to the method of Yamanaka *et al.* (29). BA and NS were enzymatically determined with commercial kits (Wako Pure Chemical Industries) as described (30).

Tissue lipolytic activities. Heparin-releasable TG lipases were obtained from epididymal fat pads (lipoprotein lipase, LPL) and liver (hepatic TG lipase, HTGL). Each of the fresh tissues weighing 300 mg was homogenized in 0.2 M Tris-HCl buffer (pH 8.5) containing 10 U/mL of sodium heparin (Wako Pure Chemical Industries) according to the method of Noguchi *et al.* (31). Lipases were incubated for 1 h in 0.2 M Tris-HCl buffer (pH 8.0 and with adequate apolipoprotein C-II as an activator of LPL for LPL assay and pH 8.5 for HTGL) containing 925 Bq/μmol of [*carboxyl*-^{14}C]triolein (925 MBq/mmol; NEN Research Products, Boston, MA), and lipase activities were estimated as described (32,33).

Statistical analysis. Statistical analyses were carried out using Student's *t*-test (normal 20-C vs. nephritic 20-C) and Duncan's multiple-range test (among the nephritic groups); a P value of <0.05 was considered significant.

RESULTS

As shown in Table 2, nephritis caused growth retardation, enlargement of the liver and kidney, and hypoalbuminemia (normal 20-C vs. nephritic 20-C). Food intake tended to be decreased by inducing nephritis. In the nephritic state, body weight gain of the 20-F and 8-C groups was comparable to that of the 20-C group, but the growth of the 8-F group was significantly suppressed when compared with that of the 20% casein (20-C, 20-F) groups. Food intake was almost the same among the four nephritic groups except for the 8-C group in which rats consumed the greatest amount. A nephritis-induced enlargement of the liver was not affected by any dietary manipulation, but that of the kidney was reduced by feeding the 8-C diet. No dietary effect was noted on the serum albumin level in any of the four nephritic groups.

While urinary protein excretion in normal rats remained constant at low levels during the experimental period, that of nephritic control (20-C) rats rapidly and linearly increased up to day 3 and remained at a high rate until day 14 (data not shown). The excretion rate of the 8% casein (8-C, 8-F) groups commenced to significantly de-

TABLE 2

Body Weight Gain, Food Intake, Tissue Weights and Serum Albumin Level[a]

Measurement	Normal	Nephritis			
	20-C	20-C	20-F	8-C	8-F
Body weight gain (g/14 d)	110 ± 4[b]	70 ± 5[c]	65 ± 7[c]	65 ± 11[c,d]	42 ± 4[d]
Food intake (g/14 d)	272 ± 7	244 ± 11[c]	237 ± 10[c]	280 ± 17[d]	233 ± 8[c]
Liver weight (g/100 g body weight)	3.8 ± 0.2[b]	4.8 ± 0.1[c]	4.6 ± 0.2[c]	4.6 ± 0.1[c]	4.6 ± 0.1[c]
Kidney weight (g/100 g body weight)	0.7 ± 0.0[b]	1.1 ± 0.1[c,d]	1.1 ± 0.1[c]	0.9 ± 0.0[d]	1.1 ± 0.1[c]
Serum albumin (g/L)	40 ± 0[b]	29 ± 2[c]	31 ± 0[c]	32 ± 1[c]	31 ± 1[c]

[a]Values are means ± SEM for six rats. Rats were treated as described in the Materials and Methods section; 20-C, a 20% casein diet containing corn oil; 20-F, a 20% casein diet containing fish oil; 8-C, an 8% casein diet, supplemented with cystine and threonine; 8-F, an 8% casein diet, supplemented with cystine and threonine, containing fish oil.
[b]Significantly different from the nephritic 20-C group at $P < 0.05$ by Student's t-test.
[c,d]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

crease on day 7, and FO, in contrast to CO, showed no lowering effect on urinary protein excretion at either the 20 or the 8% casein level (Fig. 1).

Table 3 shows serum and liver lipid levels. Hyperlipidemia characterized by elevations in Ch, TG and PL was confirmed in nephritic control rats (20-C) as compared with normal rats, and the hyperlipidemia was reduced by feeding either low casein (20-C vs. 8-C, 20-C vs. 8-F) or FO containing (20-C vs. 20-F) diets. The serum NEFA level tended to decrease upon nephritis induction, and FO feeding (20-F, 8-F) further decreased the level significantly in nephritic rats compared with CO feeding (20-C, 8-C). Induction of nephritis significantly reduced liver Ch and TG

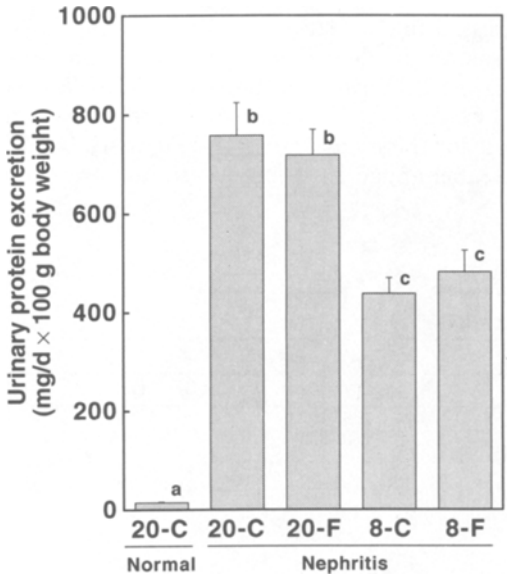

FIG. 1. Urinary protein excretion on day 7. Each value and vertical bar represents the mean of six rats and standard error, respectively. (a) Significantly different from the nephritic 20% casein diet containing corn oil (20-C) group at $P < 0.05$ by Student's t-test. (b,c) Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test; 20-F, a 20% casein diet containing fish oil; 8-C, an 8% casein diet, supplemented with cystine and threonine, containing corn oil; 8-F, an 8% casein diet, supplemented with cystine and threonine, containing fish oil.

levels. The Ch level was unchanged among the four nephritic groups. The TG level was increased, but the PL level was decreased by feeding the 8% casein diets.

As seen in Table 4, hepatic Ch synthesis accelerated by nephritis was suppressed by feeding the 8% casein diets compared with the 20% casein diets, and tended to be reduced by FO feeding between the groups at the same casein levels (20-C vs. 20-F and 8-C vs. 8-F). Although hepatic FA synthesis was significantly stimulated by inducing nephritis, no dietary effect was observed in nephritic rats. Nephritis induction did not influence fecal steroid excretion. Administration of FO to the nephritic rats (20-F, 8-F) caused a suppression of NS excretion when compared with CO (20-C, 8-C) feeding. BA excretion was significantly suppressed in the 20-F group compared with the other three groups. No significant nephritic or dietary effects were observed on epididymal adipose tissue LPL activity. Though HTGL activity was unchanged by the nephritis induction, the activity was slightly but significantly lower in the 20-F, 8-C and 8-F groups than in the 20-C group.

DISCUSSION

In the present study, we examined both the individual and the concurrent effects of dietary FO and of an 8% casein diet supplemented with cystine and threonine, which are limiting amino acids of casein, on hyperlipidemia and proteinuria in rats with NSN. Protein malnutrition as manifested in growth retardation and severe fatty liver was not seen when rats were administered the 8% casein diets supplemented with the limiting amino acids (8-C, 8-F). Proteinuria, hypoalbuminemia and hyperlipidemia were caused by nephritis induction. Proteinuria was reduced by feeding the 8% casein diets (8-C, 8-F) irrespective of the dietary oil used. The hyperlipidemia was caused, at least in part, by stimulated hepatic Ch and FA syntheses (Table 4), which is in good agreement with our own data (9) and earlier studies by others (34). A reduction of hypercholesterolemia and hypertriglyceridemia was observed in nephritic animals fed the 8-C compared with those fed the 20-C diet (Table 3). FO also had a noticable hypolipidemic effect at the 20% casein level (20-C vs. 20-F); however, at the 8% casein level, the hypolipidemic effect was no longer seen (8-C vs. 8-F and 20-F vs. 8-F). Interestingly, liver TG

TABLE 3

Serum and Liver Lipid Levels[a]

Measurement	Normal	Nephritis			
	20-C	20-C	20-F	8-C	8-F
Serum lipids (mmol/L)					
Ch	2.1 ± 0.1[b]	6.8 ± 0.7[c]	4.2 ± 0.5[d]	4.8 ± 0.5[d]	5.3 ± 0.6[c,d]
TG	0.8 ± 0.2[b]	1.7 ± 0.3[c]	0.7 ± 0.1[d]	0.9 ± 0.2[d]	0.6 ± 0.1[d]
PL	2.0 ± 0.1[b]	5.0 ± 0.5[c]	2.9 ± 0.3[d]	3.5 ± 0.1[d]	3.1 ± 0.4[d]
NEFA	0.8 ± 0.1	0.5 ± 0.1[c]	0.3 ± 0.0[d]	0.6 ± 0.0[c]	0.4 ± 0.0[d]
Liver lipids (μmol/g liver)					
Ch	3.8 ± 0.1[b]	3.3 ± 0.1[c]	3.3 ± 0.1[c]	3.4 ± 0.2[c]	3.4 ± 0.1[c]
TG	10.0 ± 1.1[b]	4.8 ± 0.6[c]	5.7 ± 2.1[c]	15.9 ± 2.7[d]	14.2 ± 3.2[d]
PL	38.9 ± 0.6	38.5 ± 1.0[c]	38.9 ± 1.6[c]	33.2 ± 2.5[d]	33.5 ± 1.0[d]

[a]Values are means ± SEM for six rats. Rats were treated as described in the Materials and Methods section; Ch, cholesterol; TG, triglyceride; PL, phospholipid; NEFA, nonesterified fatty acids. See Table 2 for other abbreviations.
[b]Significantly different from the nephritic 20-C group at $P < 0.05$ by Student's *t*-test.
[c,d]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

and PL contents were affected by the dietary casein level but not by the dietary oil type. The effect of low casein level observed here is not due to a deficiency in essential amino acid for growth, since the 8-C actually induced the same growth as the 20-C.

The hypocholesterolemic action of low casein diets may, at least in part, be due to a reduced hepatic Ch synthesis, which was stimulated by nephritis induction. In contrast, the hypotriglyceridemic action of low casein diets may be partly due to suppressed TG secretion from the liver, since the liver TG accumulated in low casein groups (8-C, 8-F) when compared with the 20% casein groups (20-C, 20-F). The hypocholesterolemic action of FO may also, at least in part, be due to reduced hepatic Ch synthesis, and the hypotriglyceridemic action of FO may partly be due to a lower FA mobilization from peripheral adipose tissue. As reported previously for normal rats, it is possible that FO may increase FA oxidation and reduce intestinal absorption of TG (35). Although the serum Ch level was lowered by FO treatment at the 20% casein level, hepatic Ch synthesis was slightly reduced and fecal steroid excretion was decreased. There have been reports that FO reduced serum Ch levels, due to a reduced secretion of Ch from the liver (10) and an increased biliary excretion of Ch compared with CO (36), and a higher binding affinity of low density lipoprotein receptors compared with coconut oil feeding (37) to normal rats. The FO effects observed in normal rats might occur in nephritic rats fed the FO diet. Interestingly, FO suppressed fecal NS excretion. It has to be considered that FO might induce changes in the gastrointestinal flora of the rats, although the cause of this suppression is unclear. The CO and FO used here are actually in the TG form, and there was little NS in both the CO and FO, so that NS in the diets are unlikely to be responsible for the results. In the present study, LPL activity was unchanged by nephritis induction, which is in

TABLE 4

Hepatic Lipogenesis, Fecal Steroid Excretion and Tissue Lipolytic Activities[a]

Measurement	Normal	Nephritis			
	20-C	20-C	20-F	8-C	8-F
Hepatic lipid syntheses (dpm × 10^{-4}/2 h/g liver)					
Ch	2.9 ± 0.7	5.5 ± 1.4[c]	4.8 ± 1.0[c,d]	2.8 ± 0.8[c,d]	1.9 ± 0.5[d]
FA	3.2 ± 0.5[b]	6.6 ± 1.1[c]	6.3 ± 1.6[c]	7.7 ± 1.3[c]	4.1 ± 0.8[c]
Fecal steroid excretion (μmol/2 d/100 g body weight)					
NS	17 ± 1	16 ± 1[c]	8 ± 1[d]	20 ± 2[c]	8 ± 1[d]
BA	9 ± 1	11 ± 1[c]	7 ± 1[d]	11 ± 1[c]	10 ± 2[c]
Tissue lipolytic activities (μmol FFA/h/g tissue)					
LPL	4.8 ± 1.0	4.3 ± 0.4[c]	4.5 ± 1.0[c]	4.2 ± 1.0[c]	4.9 ± 0.4[c]
HTGL	0.3 ± 0.0	0.3 ± 0.0[c]	0.2 ± 0.0[d]	0.2 ± 0.0[d]	0.2 ± 0.0[d]

[a]Values are means ± SEM for six rats. Rats were treated as described in the Materials and Methods section; Ch, cholesterol; FA, fatty acid; NS, neutral sterols; BA, bile acids; FFA, free fatty acid; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase. See Table 2 for other abbreviations.
[b]Significantly different from the nephritic 20-C group at $P < 0.05$ by Student's *t*-test.
[c,d]Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

agreement with the results by others (38,39). Neither low protein nor FO intake influenced LPL activity, and HTGL activity was decreased by low protein or FO feeding. It has been reported that abnormalities in lipoprotein components rather than LPL activity were observed in nephrosis (38,39). Thus, there is a possibility that abnormalities in the lipoprotein components in the present study may have been normalized by the low protein intake. Hepatic TG lipase activity was unchanged between the normal and nephritic 20-C groups, although HTGL activity was reported to decrease in nephrosis (40). The reason for this discrepancy is not clear at present. In the present study, the dietary vitamin E level was the same among four diets (350 mg/kg diet). No side effects and no effects on lipid metabolism have been noted at the dose of vitamin E used here (16).

Scharschmidt *et al.* (14) reported that FO decreased the syntheses of glomerular thromboxane (TX) A_2, a vasoconstrictor, and prostaglandin E_2, a vasodilator, and that FO had beneficial effects on NSN. They examined urinary protein excretion and insulin clearance before and 48 to 72 h after anti-GBM antiserum injection, and antiserum injection induced a low level of proteinuria (about 1.5 mg/d × rat in normal vs. 15 mg/d × rat in nephritis). The rats we used were maintained for 14 d and showed a high excretion rate for urinary protein (15 mg/d × 100 g body weight in normal vs. 758 mg/d × 100 g body weight in nephritis, Fig. 1); this suggests that the nephritis seen by Scharschmidt and her colleagues (14) was in an earlier stage than ours. Watanabe *et al.* (15) showed that high α-linolenate intake complicated symptoms of NSN compared to high linoleate intake, although a previous study by Suzuki *et al.* (19) had demonstrated that TXA_2 synthetase inhibitors improved NSN. These findings suggest that the effectiveness of vasodilatory and vasoconstrictive eicosanoids may be different depending on the stage of nephritis (15). Further experiments including survival studies and histologic analyses are needed to clarify these aspects.

In conclusion, we have shown that amino acid-fortified low casein diets improved hyperlipidemia and proteinuria without retarding growth in NSN. The hypolipidemic action of FO might be different from that of low protein diets. FO might maintain the abovementioned hypolipidemic effects in the nephritic state as well as in the normal state.

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Adipose Monoacylglycerol:Acyl-Coenzyme A Acyltransferase Activity in the White-Throated Sparrow (*Zonotrichia albicollis*): Characterization and Function in a Migratory Bird

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Although migrating birds use stored triacylglycerol as their primary fuel for flight, they must retain sufficient stores of ω6 and ω3 fatty acids to sustain reproduction after the spring migration. Hepatic monoacylglycerol:acyl-coenzyme A acyltransferase (EC 2.3.1.22) (MGAT) activity is associated with physiological periods in which lipolysis and ß-oxidation are prominent, and it may also play a role in the selective retention of certain essential fatty acids. Therefore, we characterized MGAT activity in adipose tissue from the white-throated sparrow (*Zonotrichia albicollis*), a migratory bird. MGAT specific activity from adipose tissue and liver, respectively, was 22.2 ± 7.27 and 0.79 ± 0.35 nmol/min/mg of total particulate protein. Activity did not vary seasonally or between male and female birds. Specific activity increased 4.3-fold in the presence of 75 µg of phosphatidylcholine and phosphatidylserine (1:1, w/w). MGAT acylated *sn*-1(3)-monooleoylglycerol, *sn*-2-monooleylglycerol ether and *sn*-1(3)-monooleylglycerol ether at 7.5, 5.7 and 1.7%, respectively, of the rate observed with *sn*-2-monooleoylglycerol. An initial lag phase observed at low concentrations of palmitoyl-CoA was corrected by adding 2 mM $MgCl_2$, $Mg(NO_3)_2$ or $CaCl_2$, suggesting a requirement for divalent cations. MGAT acylated *sn*-2-monolinolenoylglycerol and *sn*-2-monolinoleoylglycerol in preference to *sn*-2-monooleoylglycerol. Specificity of MGAT for *sn*-2-monoacylglycerols and the probable enhanced affinity for *sn*-2-monoacylglycerols of specific acyl chains may allow selected ω6 and ω3 fatty acids to be retained within the adipocyte, while nonessential fatty acids are released for β-oxidation in flight muscles.

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Seasonal migration is characteristic for many birds that breed at middle and high latitudes. Sparrows, small birds with relatively high metabolic rates (1), avoid winters by migrating to warmer regions. During migratory flight, replenishing energy reserves may be difficult or impossible (2–4). Thus, triacylglycerol stored in adipose tissue serves as the primary fuel for flight muscles during migration by providing fatty acids for oxidation (1,5–7).

Although studies of the metabolism and action of ω3 and ω6 fatty acids and their derivatives in migrating birds are limited, their importance in poultry is clear (8,9–13). Linoleic acid deficiency results in poor growth, reduced resistance to disease, fatty liver and reduced gonadal size. Reproductive failure, probably the result of eicosanoid deficiency, is manifested by decreased egg weight and embryo viability. The ω3 fatty acid, α-linolenic acid, is probably required for the synthesis of phospholipids in nervous tissue and retina in the developing chick (8).

When normal food supplies are absent or diminished, the massive oxidation of stored fatty acids during migration could deplete endogenous stores of ω3 and ω6 fatty acids, and lead to impaired reproductive capacity when birds arrive at their breeding sites. Studies in chickens have suggested that a mechanism exists to prevent the loss of ω3 and ω6 fatty acids from endogenous stores. In chicks, ω3 and ω6 fatty acids accumulate in tissue glycerolipids out of proportion to their relative amounts in egg yolk (9,14), and one might hypothesize that selected essential fatty acids might be similarly retained during migration in order to preserve eicosanoid precursors that are critical for reproduction. The tissue content of fatty acid species varies seasonally, but differences in fatty acid composition of foods consumed have confounded most studies of free-living and migrating birds (15–19).

We have suggested that animals that undergo normal physiological states characterized by massive lipolysis and β-oxidation might selectively retain ω3 and ω6 fatty acids, and that the monoacylglycerol pathway of glycerolipid synthesis could provide a mechanism for their retention (20). The monoacylglycerol pathway is defined by the presence of the monoacylglycerol:acyl-CoA (coenzyme A) acyltransferase (MGAT) (EC 2.3.1.22), a microsomal enzyme activity that stereospecifically acylates *sn*-2-monoacylglycerols to form *sn*-1,2-diacylglycerols (21,22). Since ω3 and ω6 fatty acids are predominantly found in the *sn*-2 position of most glycerolipids (23,24), reacylation of *sn*-2-monoacylglycerols would tend to conserve these acyl species, particularly if MGAT were selective in the monoacylglycerol species that are reacylated.

We have hypothesized that hepatic MGAT provides a mechanism by which essential fatty acids may be retained during physiological states of exaggerated lipolysis and β-oxidation. Such states occur in neonatal rats, chick embryos, fetal guinea pigs and hibernating marmot (20). The enhanced expression of hepatic MGAT activity in streptozotocin-induced diabetes in adult rats is consistent with a role for this microsomal activity in causing or maintaining the different essential fatty acid composition that characterizes membrane lipids in the diabetic animal (25).

Because migrating birds undergo a prolonged state of accelerated lipolysis in order to provide fatty acids to power flight muscles, we wondered whether migration was associated with changes in MGAT activity in adipose

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Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; CoA, coenzyme A; EDTA, (ethylenedinitrilo)tetraacetic acid; MGAT, monoacylglycerol acyl-coenzyme A acyltransferase; NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); PC, phosphatidylcholine; PS, phosphatidylserine; Tris-HCl, (hydroxymethyl)aminomethane hydrochloride.

tissue or liver. We found very high MGAT activity in adipose tissue, but not in liver, of the white-throated sparrow. In order to determine whether adipose MGAT activity might play a role in the selective retention of essential fatty acids in migrating birds, we characterized the activity and tested its ability to utilize monoacylglycerols that contain different acyl groups.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC; from egg lecithin), phosphatidylserine (PS; from beef brain), *sn*-2- and *sn*-1(3)-monooleoylglycerols, *sn*-1(3)- and *sn*-2-monooleylglycerol ethers, trilinolenin and trilinolein were obtained from Serdary Research Laboratories, Inc. (London, Ontario, Canada). Bovine serum albumin (essentially fatty acid-free) and Type VI-S porcine pancreatic lipase were from Sigma (St. Louis, MO). Cytoscint was from ICN Pharmaceuticals (Cleveland, OH). [^{3}H]Palmitic acid was obtained from New England Nuclear (Boston, MA). [^{3}H]Palmitoyl-CoA was synthesized enzymatically (26). Scored 250 micron silica gel G and plates impregnated with 10% $AgNO_3$ or with 3% borate were from Analtech, Inc. (Newark, DE).

Synthesis of monoacylglycerols. sn-2-Monolinoleoylglycerol and *sn*-2-monolinolenoylglycerol were synthesized from trilinolein and trilinolenin, respectively, by incomplete enzymatic hydrolysis with Type VI-S procine pancreatic lipase (27). The reaction products were extracted with diethyl ether, and the *sn*-2-monoacylglycerols were separated from 1(3)-monoacylglycerols and other lipids on 3% borate silica gel G plates developed with chloroform/acetone/methanol/acetic acid (90:5:2:0.5, by vol). Monoacylglycerols were stored in hexane at -20°C until use within a few days of synthesis. Butylated hydroxytoluene (0.05%) was present during enzymatic hydrolysis and storage. The *sn*-2- and *sn*-1(3)-monoacylglycerols were quantified colorimetrically (28). To minimize acyl migration, commercially obtained monoacylglycerols were kept in small aliquots at -20°C.

Collection and preparation of tissue samples. White-throated sparrows (*Zonotrichia albicollis*) arrive from their breeding localities in southern Ontario by late October and remain in North Carolina until the onset of their vernal migration in early April. Birds were captured in Durham, North Carolina. At Duke University, Durham, each sparrow was caged individually. A natural light/dark photoperiod was maintained. The laboratory diet consisted of 70% canary and 30% large black rapeseeds (G.W. Hill Co., Inc., Florency, KY) and was supplemented with worms and hard-boiled eggs with shells. Preparations of liver and fat were obtained within 5 d of capture from seven birds and at 5, 7 and 10 mon after capture of five birds that had been maintained in cages. Liver and adipose tissue fatty acids were not measured because of the inconsistencies in the bird's previous diets (natural and laboratory). Birds (about 25 g) were anesthetized with methylflurane (metaphane) and dissected. Adipose tissue was collected from the furcular (clavicule-coracoid) fossa and abdominal depots from all birds (n = 12), and liver was obtained from seven birds. Fat depots were minimal in July and November (four samples), low in January (four samples), and greatest in April and May (four samples). Tissue was homogenized in Medium I consisting of 0.25 M sucrose, 1 mM (ethylenedinitrilo)tetraacetic acid (EDTA), 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-Cl), pH 7.4 in a Teflon-glass homogenizing vessel with 10 up and down strokes at medium speed. Total particulate preparations were pelleted by centrifuging each homogenate in Medium I at 100,000 × *g* for 1 h. Protein was measured by the method of Lowry *et al.* (29) using bovine serum albumin as standard.

Assay of MGAT activity. MGAT activity was assayed at 23°C under conditions that measured initial rates, in a final volume of 200 µL, by modifying a previously described method (22). The reaction mixture contained 100 mM Tris-Cl, pH 8.0, 1 mg/mL bovine serum albumin, 25 µM [^{3}H]palmitoyl-CoA and 50 µM *sn*-2-monooleoylglycerol dispersed in acetone. The final acetone concentration was 2.5%. The reaction was initiated by adding 2 to 6 µg of total particulate protein and was terminated after 5 min by adding 1.5 mL of propanol-2/heptane/water (80:20:2, by vol) and mixing on a Vortex mixer. After adding 1 mL heptane and 0.5 mL water, the heptane layer was removed and washed twice with 2 mL 0.5 N NaOH/ethanol/water (10:50:50, by vol). Aliquots of the final heptane phase were counted in 4 mL of Cytoscint. The remaining portion of the heptane phase was concentrated in a Savant Speed Vac concentrator and chromatographed with carrier lipids on 10-cm silica gel G plates using heptane/isopropyl ether/acetic acid (60:40:4, by vol). Lipids were made visible by exposure to I_2 vapor, and the bands corresponding to diacylglycerol and triacylglycerol were scraped off and counted. MGAT specific activities were calculated by subtracting one-half the counts per minute which appeared in triacylglycerol.

Monoacylglycerol specificity. Total particulate preparations from sparrow adipose tissue (2.5 µg protein) were incubated with 20 µM each of 2-monooleoylglycerol and 2-monolinolenoylglycerol. Diacylglycerols and triacylglycerols were separated on silica gel G plates using heptane/isopropyl ether/acetic acid (60:40:4, by vol), and were eluted with $CHCl_3$. The mono- and triunsaturated diacylglycerols and triacylglycerols were separated on 10% $AgNO_3$-impregnated plates using hexane/diethyl ether (15:85, vol/vol) for diacylglycerols and (70:30, vol/vol) for triacylglycerols (30).

RESULTS

MGAT activity. In order to determine whether MGAT activity in migrating birds might play a role in the selective retention of essential fatty acids, we examined hepatic and adipose tissue MGAT activity in the white-throated sparrow. All studies were performed under conditions that measured initial rates. No differences were observed in the activities between males and females or in birds that had been kept in captivity compared to those studied immediately after capture. MGAT specific activity was 22.2 ± 7.27 nmol/min/mg of protein (range 9.3–34) in total particulate fractions from twelve independent preparations of sparrow adipose tissue. Even though MGAT was as-

sayed in total particulate fractions from whole adipose tissue, the mean activity found was 5-fold higher than the activity comparably assayed in rat adipose tissue microsomes (31). In liver from seven independent preparations, MGAT specific activity was 0.79 ± 0.35 nmol/min/mg of protein (range 0.39–1.35). The low hepatic activity was similar to that observed in adult rat liver (21). Despite seasonal changes in the amount of adipose tissue, the specific activities of MGAT in both fat and liver did not change (data not shown).

Because our previous studies suggested that the high MGAT activity observed in liver under conditions of enhanced lipolysis might be related to selective retention of ω3 and ω6 fatty acids, the high adipose MGAT activity was characterized further. The activity was proportional to the concentration of protein employed up to 10 μg at 5 min and with time to 11.5 min when 2 μg of protein was used (data not shown). More than 96% of the labeled product was identified by thin-layer chromatography as diacylglycerol and triacylglycerol. Unlike the products of MGAT activity from rat (21) and chick liver (32), the relative amounts of diacylglycerol and triacylglycerol products varied minimally with time; at each time point, about 40% of the product was diacylglycerol and about 50% was triacylglycerol (data not shown).

Effect of phospholipids and bovine serum albumin. Addition of 0.3 mg/mL of PC/PS (1:1, w/w) stimulates rat intestinal and neonatal hepatic MGAT activities as much as 4- and 2-fold, respectively (22), but inhibits the activity from adult liver (25). In sparrow adipose tissue, a maximal 4.3-fold increase was observed with 0.375 mg/mL of the PC/PS mixture (Fig. 1).

The effect of bovine serum albumin on adipose tissue MGAT activity was examined because the activity is stimulated in its presence in microsomes from liver and intestinal mucosa (22) and rat adipocytes (31). In sparrow adipose tissue, however, the addition of up to 1.0 mg/mL bovine serum albumin did not affect adipose tissue MGAT activity. Bovine serum albumin inhibited MGAT activity about 15% at 1.25 mg/mL (data not shown).

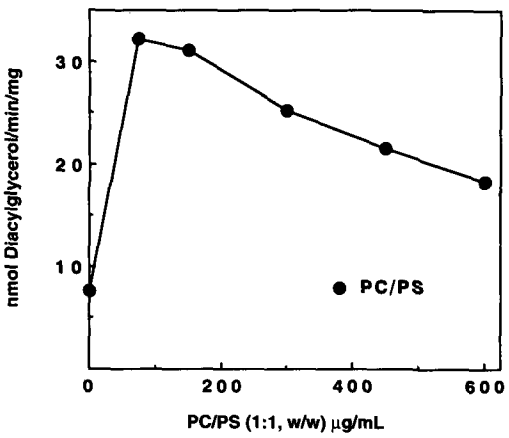

FIG. 1. Dependence of monoacylglycerol:acylCoenzyme A acyltransferase activity on phosphatidylcholine/phosphatidylserine (PC/PS) (1:1, w/w). Assays were performed with 2 μg of protein as described in the Materials and Methods section except that the amount of added phospholipid was varied as indicated. Without added PC/PS, the specific activity was 12.7 nmol/min/mg. This figure is representative of two independent studies using different preparations.

Monoradylglycerol specificity. The hepatic MGAT activity from suckling rats and chick embryos is highly specific for *sn*-2-monoacylglycerols and acylates monoalkylglycerol ethers poorly. In contrast, MGAT activity from rat intestine readily acylates monoalkylglycerols, perhaps for the purpose of allowing monoalkylglycerol ethers from the diet to enter chylomicra triradylglycerols (33). To determine whether the MGAT activity of sparrow adipose tissue shared the specificity of that of the liver, we tested its ability to acylate various monoradylglycerols. The activity from adipose tissue was highly specific for *sn*-2-monoacylglycerols and had a diminished ability to acylate either 1(3)-monoacylglycerol or monoalkylglycerols. At a concentration of 50 μM of each of the substrates, MGAT acylated 1(3)-monooleoylglycerol, *sn*-2-monooleylglycerol ether and 1(3)-monooleylglycerol ether at 7.5, 5.7 and 1.7%, respectively, of the rate observed with *sn*-2-monooleoylglycerol. These results suggest that the MGAT activity of adipose tissue, like that of liver, is highly selective in its substrate specificity. In fact, the low activity observed with 1(3)-monoacylglycerol may have been due to the presence of small amounts of *sn*-2-monoacylglycerol due to acyl migration.

We have previously shown that hepatic MGAT from three different species has a greater affinity for *sn*-2-monoacylglycerols that contain 18:2 or 18:3 fatty acids than for those containing 18:1 or 20:4 (20). In order to determine whether MGAT from sparrow adipose tissue exhibits a similar acyl-chain preference, we tested its ability to acylate monoacylglycerols that contain different fatty acids at the *sn*-2 position. The enzyme activity seen with *sn*-2-18:1-glycerol monoester was compared with the activity seen with the more unsaturated *sn*-2-18:2 and *sn*-2-18:3-glycerol monoesters (Fig. 2). We found that MGAT acylated *sn*-2-18:3-glycerol monoester and *sn*-2-18:2-glycerol monoester in preference to *sn*-2-18:1-glycerol monoester. An increased rate of acylation of *sn*-2-18:2 and *sn*-2-18:3-glycerol monoesters was observed at each of the concentrations of monoacylglycerols examined. Although the apparent K_m values were 20, 47 and >1000 μM for *sn*-2-18:3-glycerol monoester, *sn*-2-18:2-glycerol monoester and *sn*-2-18:1-glycerol monoester, respectively, Michaelis-Menten kinetics cannot be universally applied to this microsomal system with hydrophobic and amphipathic substrates. The apparent K_m value for *sn*-2-18:1-glycerol monoester with MGAT from liver was <50 μM (21,32). We hypothesized that the preference shown by MGAT for *sn*-2-acylglycerols would tend to ensure the relative conservation of essential fatty acids which predominate in the *sn*-2 position of glycerolipids (20). In addition, the apparent increase in affinity for *sn*-2-acylglycerols containing 18:2 and 18:3 may enable MGAT to play a role in conserving specific ω3 and ω6 fatty acids during periods of accelerated lipolysis. The increased apparent affinity is particularly evident at low monoacylglycerol concentrations. Low amounts of monoacylglycerol are likely to be present normally in the endoplasmic reticulum (20). Although the concentration of *sn*-2-monoacylglycerol in

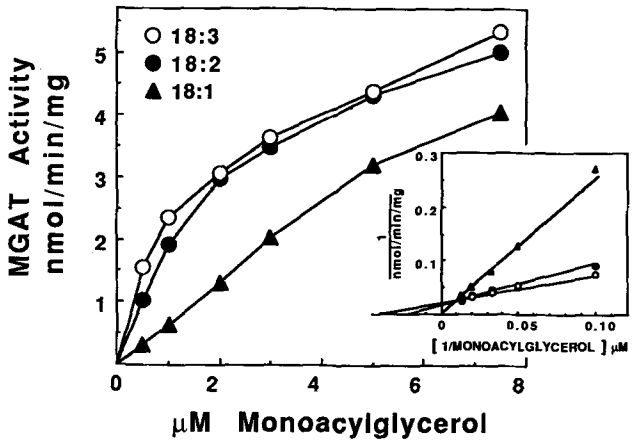

FIG. 2. Dependence of monoacylglycerol acyltransferase activity on *sn*-2-monoacylglycerols with different acyl chains. Each monoacylglycerol substrate was added in 5 μL acetone as described in the Materials and Methods section. The amount of total particulate protein present in each assay was 2.5 μg. The inset shows the double reciprocal plot of the data (Ref. 34). The regression coefficients were all >0.996. With 50 μM *sn*-2-18:1-glycerol monoester, the specific activity was 21.0 nmol/min/mg. Each point is the average of duplicate determinations that varied by less than 5%.

adipocyte endoplasmic reticulum is not known, we have calculated that, in liver, the normal concentration would be less than 1 mol% or about 2 μM (20). This concentration is well within the range at which MGAT exhibits differential affinity for monoacylglycerols having different acyl chains (Fig. 2).

To test directly the effect of the more unsaturated species on MGAT activity, 20 μM each of *sn*-2-18:1- and 18:3-glycerol monoesters were added to the assays, and the products were resolved by argentation chromatography according to their degree of unsaturation (30). Analyses of the products indicated that MGAT acylated 39.5% of the *sn*-2-monooleoylglycerol and 60.5% of the *sn*-2-monolinolenoylglycerol, consistent with a preference for the more unsaturated monoacylglycerols.

Dependence on palmitoyl-CoA and the effect of divalent cations and salts. The dependence of sparrow adipose tissue MGAT on palmitoyl-CoA showed an initial lag phase between 5 and 20 μM, was concave upward between 20 and 40 μM, and reached a plateau at about 40 μM (Fig. 3). The sigmoidal shape of the palmitoyl-CoA dependence curve is typical of allosteric enzymes which are regulated by specific effector molecules (35–37). To determine whether any of a variety of possible effectors might eliminate the lag in the dependence curve, we examined the effect of adding various possible effectors to the enzyme assay. Addition of 5 mM adenosine triphosphate (ATP), 2 mM adenosine monophosphate (AMP), 1 mM nicotinamide adenine dinucleotide (NAD), 1 mM nicotinamide adenine dinucleotide (reduced form) (NADH), 1 mM CoA, or 1 mM citrate to the assay mixture had no effect on the initial lag period. However, the lag phase observed at 5 and 10 μM palmitoyl-CoA was corrected by adding 2 or 10 mM $MgCl_2$, $Mg(NO_3)_2$ or $CaCl_2$ (Fig. 3). Although, in each case, 10 mM salt was more stimulatory, each of the

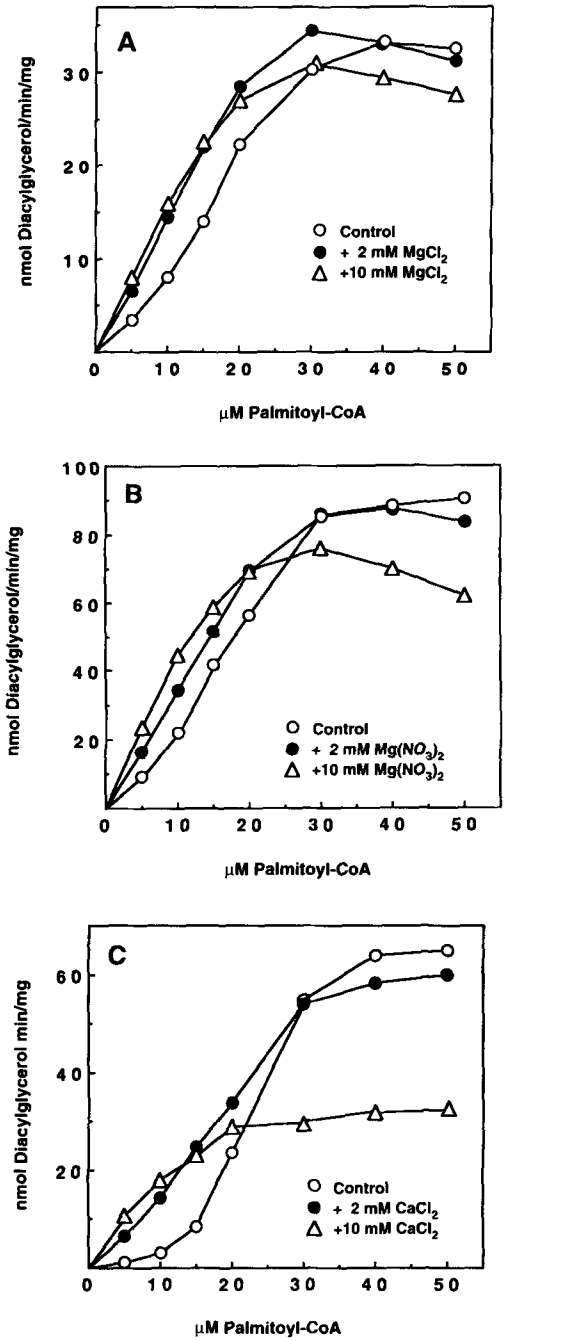

FIG. 3. Dependence of monoacylglycerol acyltransferase activity on palmitoyl-coenzyme A in the absence or presence of A) $MgCl_2$, B) $MgNO_3$, and C) $CaCl_2$. Divalent cations were present in the assay mixture at 0, 2, or 10 mM as indicated. Each of the experiments used a different adipose tissue preparation. The study with $MgCl_2$ was representative of two other studies using independent adipose tissue preparations.

salts at 10 mM inhibited MGAT activity when the palmitoyl-CoA concentration was greater than 20 to 30 μM. These results suggested a requirement for divalent cations when a low concentration of the palmitoyl-CoA substrate was present. The lack of a lag at higher concentrations of palmitoyl-CoA was unlikely to be due to a con-

taminating salt in the palmitoyl-CoA preparation itself since the addition of 10 mM EDTA did not alter the palmitoyl-CoA dependence at any of the concentrations of palmitoyl-CoA (data not shown).

A number of enzymes are activated by incubation with Mg^{2+}, unrelated to an ATP cofactor requirement (35–37). Enzyme activation by metal ions may be related to polymerization, to a change in enzyme conformation, to involvement in enzyme catalysis or to an alteration in substrate binding. Since MGAT has not been purified to homogeneity, mechanistic conclusions cannot be drawn. However, in order to determine whether the divalent cations might be exerting their effect by stabilizing MGAT, we tested the ability of $MgCl_2$ to enable MGAT activity to withstand thermal denaturation. With either 10 or 40 μM palmitoyl-CoA present, MGAT activity decreased 62% after 4 min of heating at 56°C in both the presence or absence of 2 or 10 mM $MgCl_2$ (data not shown). These results suggest that Mg^{2+} does not enhance activity by stabilizing the enzyme in a functional state.

We tested a variety of salts at 2.5 and 10 mM in order to determine whether other divalent cations and salts stimulated MGAT activity. The palmitoyl-CoA concentration was maintained at 10 μM (Table 1). MGAT activity was stimulated greatly in the presence of Mg^{2+}, Ca^{2+} and Ba^{2+}, was minimally stimulated by Na^+, was unaffected by Li^+, and was inhibited by Co^{2+}, Fe^{2+} and Zn^{2+}. $CuCl_2$ stimulated MGAT activity at 2.5 mM and inhibited it at 10 mM. Thus, the stimulatory effect was not universal for divalent cations or salts.

Previous studies of MGAT from liver have not shown an initial lag in the palmitoyl-CoA dependence, nor was MGAT stimulated by the addition of salts to the enzyme assay (21,32). In the presence of 25 μM palmitoyl-CoA, $CaCl_2$ and $MgCl_2$ inhibited MGAT activity. In a detailed study of MGAT from rat adipocytes, however, 5 mM $MgCl_2$, $CaCl_2$ or $BaCl_2$ stimulated MGAT activity when an optimum concentration (25 μM) of palmitoyl-CoA was present (31).

TABLE 1

Effect of Different Metal Ions on MGAT Activity at Low Palmitoyl-CoA Concentrations[a]

	Percent of control					
Concentration	$MgCl_2$	$MgSO_4$	$Mg(NO_3)_2$	$CaCl_2$	NaCl	Ba acetate
2.5 mM	276	318	369	384	104	183
10.0 mM	543	541	573	491	137	307

	Percent of control					
Concentration	LiCl	$CoCl_2$	$CuCl_2$	$FeCl_3$	$FeSO_4$	$ZnCl_2$
2.5 mM	83	80	114	11	6	3
10.0 mM	120	21	22	3	1	16

[a]Assays were performed as described in the Materials and Methods section except that 10 μM [^{3}H]palmitoyl-coenzyme A (CoA) was used and salts were present in the incubation mixture at 2.5 or 10 mM, as indicated. All assays were performed on the same day using 2 μg protein from the same enzyme preparation. The control monoacylglycerol acyl-coenzyme A acyltransferase (MGAT) specific activity with 10 μM palmitoyl-CoA was 1.16 nmol/min/mg. Results shown are the averages from duplicate assays. Two other enzyme preparations gave qualitatively similar results.

DISCUSSION

Our data show that the level of MGAT specific activity in sparrow adipose tissue is similar to that of liver from suckling rat, marmot, and chick embryo (20,21,32). In these animals, the presence of high hepatic MGAT activity correlates with developmental periods in which β-oxidation of fatty acids provides the primary source of the animal's energy while, concomitantly, essential fatty acids must be retained for normal development.

As previously reported for other nonpurified MGAT activities, a mixture of PS and PC markedly stimulated adipose MGAT activity (21,32). In Triton X-100/phospholipid/mixed micelles, very low concentrations of anionic phospholipids are known to stimulate partially purified MGAT from rat liver as much as 11-fold (38). Full activation requires about 11 molecules of PS per micelle (38). The data on sparrow fat suggest that adipose MGAT may have similar requirements for anionic phospholipids.

In contrast to other reported MGAT activities, the sparrow activity's dependence on palmitoyl-CoA showed an initial lag that could be abolished by adding one of several divalent cations to the reaction mixture. Magnesium, calcium, and barium were effective activators. Although this type of response is typically observed with allosterically regulated enzymes, conclusions about MGAT's mode of regulation must await the availability of a purified enzyme preparation. In contrast to the divalent ion activators, sodium and lithium had little or no effect, and cobalt, iron (Fe^{2+}), and zinc were inhibitory.

At low concentrations of the *sn*-2-monoacylglycerol substrate, *sn*-2-monolinoleoyl- and *sn*-2-monolinolenoylglycerol were acylated in sparrow fat more readily than *sn*-2-monooleoylglycerol. We have calculated that the concentration of monoacylglycerol in hepatic membranes is about 2 μM, well within the range in which MGAT exhibits a preference for the more unsaturated species (20). This preference is not the result of changes in membrane fluidity conferred by the more unsaturated monoacylglycerols because, when incubated with equal amounts of *sn*-2-monooleoylglycerol and *sn*-2-monolinolenoylglycerol, twice as much of the 18:3 species was acylated. Similar findings were reported for three hepatic MGAT activities (20). Taken as a whole, the data suggest that MGAT may play a physiological role in helping to retain essential fatty acids within these tissues.

During migration, lipids serve as the primary fuel for flight muscles (1,39–41). Among various species of migrating birds, the amount of stored fat correlates roughly with the distance migrated and with the speed of migration (1,42). Seasonal differences in the fatty acid species in avian adipose tissue have been attributed both to the fatty acid composition of available foods, including foods consumed during the migration itself (15,18) and to innate metabolic processes (16,17,19). Although the reported increase in unsaturated species in some migrating birds was attributed to a need for greater mobility of lipid stores (42), there is little evidence that saturated fatty acid

species are mobilized poorly from adipose depots. Although the relative content of linoleate and linolenate in goose adipose triacylglycerol changed very little during migration, the content of these fatty acids after reproduction differed markedly in male and female geese, suggesting that unsaturated fatty acids were used in egg production (18).

The roles of ω6 and ω3 fatty acids in physiology, development and metabolism are less well understood in avian than in mammalian tissue. However, one would anticipate, during spring migration, that birds need to retain ω6 and ω3 fatty acids because these fatty acids will be required for reproduction and for the formation of membranes of the retina and brain in the developing chick (8–13). The question arises how ω6 and ω3 fatty acids can be retained in the face of high ongoing rates of lipolysis and β-oxidation. We hypothesize that, unlike other species in which physiologic periods of lipolysis are associated with high MGAT activity in liver (20), the migrating bird must recapture the ω6 and ω3 fatty acids before they leave the adipose stores. In this way, ω6 and ω3 fatty acids can be selectively retained within the adipocyte. Since ω6 and ω3 fatty acids are predominantly found in the *sn*-2 position of glycerolipids, including triacylglycerols (23,24), high MGAT activity in adipocytes would allow reacylation of *sn*-2-monoacylglycerols and, thereby, prevent loss of ω6 and ω3 fatty acids as muscle fuels. The increased affinity for monoacylglycerols that contain ω6 and ω3 may provide a further mechanism for selective retention.

Lipolysis and reesterification occur continuously in adipocytes, even under conditions when lipolysis is not hormonally stimulated. Under nonstimulated conditions in human adipocytes, as much as 40% of triacylglycerol is reesterified within adipocytes (43). Thus, during the nonmigratory state, adipose MGAT may also reacylate monoacylglycerols. This process might be particularly prominent during premigratory fattening and hyperphagia. After human adipocytes are stimulated by β-adrenergic drugs, as much as 8–21% of triacylglycerol is reesterified within adipocytes (43). Thus, during migration, although hormone-sensitive lipase is reesterified within adipocytes (43). Thus, during migration, although hormone-sensitive lipase is stimulated to hydrolyze adipose triacylglycerol, MGAT may provide a means of recycling *sn*-2-monoacylglycerols.

Figure 4 shows possible pathways of fatty acid metabolism in the sparrow. We hypothesize that the flux of specific fatty acids is regulated within adipose tissue. There, the specificity of adipose MGAT for *sn*-2-monoacylglycerols and the enhanced affinity for 2-monoacylglycerols that contain specific acyl chains, would allow adipocytes to retain selected ω6 and ω3 fatty acids. Other, predominantly nonessential fatty acids would be released for β-oxidation in flight muscles. Although liver is also likely to take up some fatty acid and reesterify it, MGAT activity is relatively low in adult sparrow liver, and little further conservation of ω6 and ω3 fatty acids would occur.

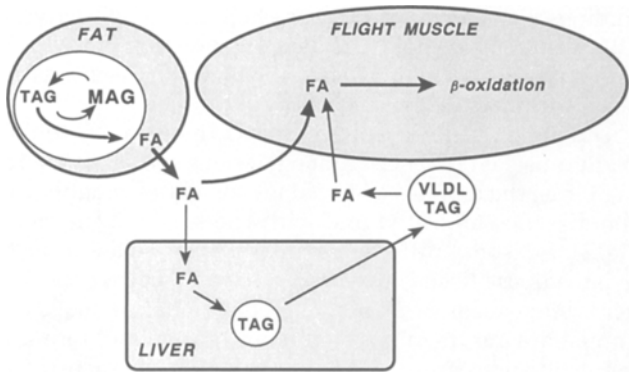

FIG. 4. Postulated pathways of avian fatty acid (FA) metabolism during migration. VLDL, very low density lipoproteins; MAG, monoacylglycerol; TAG, triacylglycerol.

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Structural and Functional Role of Lipids in Yeast and Mycelial Forms of *Candida albicans*

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The levels of total lipids, sterols and phospholipids were found to be significantly higher in the mycelial form (log phase) of *Candida albicans* than in the yeast form. Increased phospholipid levels in the mycelial form were due to higher levels of phosphatidylcholine, phosphatidylserine and phosphatidylinositol. Analyses of fatty acid composition also revealed higher levels of myristic acid (40%) in the yeast form, resulting in higher levels of saturated lipids than in the mycelial form. The changes in the lipid composition were also manifested in altered thermotropic phase behavior as gel-to-liquid crystalline phase transitions were observed at 36 and 27°C for the lipids of the yeast and mycelial forms, respectively. These changes coincided with higher uptake rate, i.e., Km and Vmax values, for the transport of L-proline and with a higher sensitivity of the mycelial form against antifungal drugs.

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Candida albicans, a fungal pathogen responsible for candidiasis, exists either as yeast or as filamentous mycelia. Both growth forms are found in tissues affected by candidiasis, but the mycelial form is considered to be more pathogenic (1). Because lipids play a role in pathogenicity and are the potential targets for many antifungal drugs, immense interest has been generated in the diverse effects of lipids in respect to membrane organization, permeability properties and sensitivity toward antifungal drugs (2–8). Studies on the lipid composition of the dimorphic forms of *C. albicans* are particularly important as the pathogenicities of the two morphological forms differ (1).

The objective of the present investigation was to compare the lipid composition and its effect on membrane transport, gel-to-liquid crystalline phase transition and sensitivity toward antifungal drugs.

MATERIALS AND METHODS

Organism and culture conditions. Candida albicans 3153, obtained from the Mycological Reference Laboratory (Central Public Health Laboratory, Colindale, London, England), was maintained in Sabouraud glucose broth and cultivated in defined mineral salt solution containing (per liter) 8 g $(NH_4)_2SO_4$, 3.6 g KH_2PO_4, 1.2 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.1 µg D-biotin on a gyratory shaker (200 rpm, Model G2; New Brunswick Scientific Co. Inc., Edison, NJ) at 37°C. Yeast and mycelial forms were obtained at pH 4.5 and 6.8, respectively. Cells were harvested in mid log phase (16-h growth) and processed further as follows.

Quantitation of lipids. Lipids were extracted by the method of Folch *et al.* (9). Total lipids were measured gravimetrically. Phospholipids were quantified by the method of Marinetti (10). Individual phospholipids were separated by one-dimensional thin-layer chromatography on Silica Gel H (11) impregnated with 0.18% ammonium sulfate in chloroform/methanol/7 N aqueous ammonia (65:25:4, by vol). Standard phospholipid markers were run side-by-side with samples, and fractions were visible by exposure to iodine vapors and identified by spraying with specific reagents (11). Phospholipid classes were quantified by phosphorus assay according to the method of Marinetti (10). Sterols were estimated by the method of Zlatkis *et al.* (12). Phospholipids were separated from total lipids by acetone precipitation, and fatty acid methyl esters were prepared as described previously (13). Fatty acid methyl esters were then analyzed by gas–liquid chromatography in an AIMIL-Nucon gas chromatograph (Delhi, India) fitted with a glass column (1.83 m × 3 mm i.d.) packed with 15% diethyleneglycolsuccinate on 80–100 mesh. Fatty acids were identified by comparison of their retention times with those of authentic standards. Peak areas were quantified by triangulation.

Enzyme assays. The activity of glycerol kinase was determined as described earlier (14). The assay for the activity of glycerol-3-phosphate acyltransferase was carried out as described by Okuyama *et al.* (15). For the separation of the product, the filter disc method of Goldfine (16), as modified by van den Bosch and Vagelos (17), was used. The homogenate for the acyltransferase assay was prepared by harvesting and sonicating the cells at 4°C in 40 mM Tris-HCl buffer, pH 7.5. For the glycerol kinase assay, the homogenate was prepared in 10 mM Tris-HCl buffer, pH 8.0. The homogenate was centrifuged at 5000 × *g* for 20 min to remove cell debris, and the supernatant was used for the assays.

Uptake studies. Log phase cells were washed with 0.85% NaCl and resuspended in medium under sterile conditions. Cells were preincubated at 37°C for 30 min with shaking. Ten µCi of [^{14}C]acetate/g of cells was then added in 50 mL of medium, and at different time points 3 mL of sample from each culture was transferred into tubes containing 0.5 mL 1 M KCN (18). The tubes were centrifuged at 2700 × *g* for 15 min, the cell pellet was recovered and lipids were extracted. Radioactivity was counted in an LKB Wallace liquid scintillation counter (Turku, Finland) using a toluene-based scintillation fluid.

Liposome preparation. Liposomes were prepared by the method of Alving *et al.* (19). Briefly, lipids, extracted from log phase yeast and mycelial forms, were dissolved separately in chloroform/methanol (2:1, vol/vol) and transferred to pear-shaped flasks. Under a gentle flow of nitrogen, the solvent was evaporated to dryness to form a thin lipid film on the walls of the flasks. The flasks were then kept in a high vacuum dessiccator for 2 h to remove resid-

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; LPC, lysophosphatidylcholine; MIC, minimum inhibitory concentration; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

ual organic solvents. Liposomes were prepared by dispersing the lipids in 10 mM Tris-HCl buffer, pH 7.4, containing 145 mM NaCl and 0.025% ethylenediaminetetraacetate, followed by vortexing for 10 min in the presence of glass beads. To the final liposome preparation, 1,6-diphenyl-1,3,5-hexatriene (DPH) was added to obtain a phospholipid to probe ratio of 750:1. The final DPH concentration was 1 μM.

Steady-state fluorescence polarization. Fluorescence polarization was measured on a Kontron (Zürich, Switzerland) SFM-25 fluorescence spectrofluorimeter (excitation wavelength, 360 nm; emission wavelength, 430 nm). Polarization values (P) were determined as described earlier (20):

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} - I_{\perp}} \quad [1]$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of parallel and perpendicular polarized light, respectively. The standard deviation of polarization values was 0.02. Temperature was maintained within ±0.5°C.

Preparation of spheroplasts. Spheroplasts were prepared according to the method of Jayakumar *et al.* (21). Formation of spheroplasts was monitored microscopically. The incubation mixture was centrifuged at 1,000 × *g* for 10 min, and the supernatant containing the cell wall degrading enzymes was discarded. The pellet was washed twice with the buffer, and the spheroplasts were purified by centrifugation on a Ficoll-opaque gradient (Uppsala, Sweden) at 400 × *g* for 10 min. The purified spheroplasts that were free of cell debris recovered from the supernatant were used for further studies.

1-Anilinonaphthalene-8-sulfonate (ANS) binding studies. A fluorescent probe, ANS, was used for determining the number of binding sites in membranes of yeast and mycelial spheroplasts. The assay mixture consisted of a total volume of 2.0 mL with 10 mM citrate phosphate buffer, pH 6.5, containing 0.7 M sodium chloride, 50 μM ANS and 50–200 μg spheroplast protein. Fluorescence emission was recorded on a Kontron SFM-25 spectrofluorimeter. The numbers of binding sites were calculated from Scatchard plot analyses (22).

Transport assay. Spheroplasts (150–200 μg protein/mL) were preincubated at 37°C for 10 min after addition of cycloheximide (200 μg/mL) to inhibit protein synthesis. The reaction was initiated by addition of L-[^{3}H]proline (sp. act., 24 Ci/mmol). At the time points indicated, 0.1-mL aliquots were removed and added to 5.0 mL of chilled normal saline. The diluted suspension was rapidly filtered through 0.45 μm millipore filters, and radioactivity retained was counted in an LKB liquid scintillation counter using a toluene-based scintillation fluid.

Protein estimation. Protein was measured by the method of Lowry *et al.* (23).

Determination of minimum inhibitory concentration (MIC) of antifungal drugs. MIC values for amphotericin B, nystatin and miconazole were determined for yeast and mycelial forms of *C. albicans* over a 48-h growth period at 37°C using the standard agar dilution method. MIC was the concentration of the drug that inhibited growth by more than 90%. Stock solutions of these drugs were prepared in dimethylsulfoxide.

Statistical analysis. Student's t-test was used to assess levels of significance. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Total lipid, phospholipid and sterol contents of log phase (16-h growth) mycelial cells were significantly higher than those of yeast cells. Lipids constituted 3.8 and 4.3% of the dry weight of the yeast and mycelial forms, respectively. The mycelial forms contained significantly higher levels of phosphatidylcholine (PC), phosphatidylserine, phosphatidylinositol and lysophosphatidylcholine (LPC), and lower levels of phosphatidylethanolamine (PE) compared to the yeast form (Table 1). PC levels increased to 104% while PE levels decreased to 33% in the mycelial form as compared to those in the yeast form, suggesting that the PC in the mycelial form may possibly be formed by the methylation pathway.

Analysis of the fatty acids of the total phospholipids revealed higher levels of saturated fatty acids, particularly of myristic acid (14:0) in the yeast form, and the mycelial phospholipids were found to contain higher levels of palmitic acid (16:0), palmitoleic acid (16:1) and linoleic acid (18:2). The higher levels of saturated fatty acids in the yeast form could cause a decrease in membrane fluidity which, in turn, may affect permeability properties. The ratio of unsaturated to saturated fatty acids was about threefold higher in the mycelial form of *C. albicans* than it was in the yeast cells, similar to what had been observed by Ghannoum *et al.* (24).

Incorporation of [^{14}C]acetate as precursor of lipid biosynthesis was linear up to four minutes, and was significantly higher in the mycelial form (data not presented). Phospholipid biosynthesis in the yeast form of *C. albicans*

TABLE 1

Lipid Composition of Log Phase Cells of Yeast and Mycelial Forms of *Candida albicans* ATCC 3153[a]

Lipids	(mg/g dry wt) Yeast form	Mycelial form	Percentage change[d]
Total lipids	37.67 ± 1.53	42.76 ± 1.24[b]	13.5
Total phospholipids	9.84 ± 0.61	14.82 ± 0.21[c]	50.6
Total sterols	4.90 ± 0.20	8.07 ± 0.81[c]	64.7
Lysophosphatidylcholine (LPC)	0.91 ± 0.05	1.46 ± 0.04[c]	60.4
Phosphatidylserine	1.12 ± 0.16	1.60 ± 0.05[b]	42.9
Phosphatidylinositol	0.91 ± 0.16	1.55 ± 0.07[b]	70.3
Phosphatidylcholine (PC)	3.02 ± 0.04	6.16 ± 0.25[c]	104.0
Phosphatidylethanolamine (PE)	1.94 ± 0.06	1.31 ± 0.09[c]	32.5
Cardiolipin	0.88 ± 0.07	0.88 ± 0.10	Nil
Unknown phospholipids	1.33 ± 0.07	1.75 ± 0.12	31.5
(LPC + PC)/PE ratio	2.03	5.82	

[a]Values are mean ± SD of three different batches.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]Percentage change in mycelial lipids as compared to yeast form.

TABLE 2

Specific Activities of Phospholipid Biosynthetic Enzymes in Yeast and Mycelial Forms of *Candida albicans*[a]

Enzyme	Specific activity (nmol/mg protein/h)	
	Yeast form	Mycelial form
Glycerol kinase	7.47 ± 0.40	11.23 ± 0.40[b]
Glycerol-3-phosphate acyltransferase	5.10 ± 0.76	8.90 ± 0.80[b]

[a]Values are mean ± SD of four different batches.
[b]$P < 0.01$.

had been studied earlier (25), but no report is available so far on the activities of phospholipid enzymes in the mycelial form. Our data revealed that the activities of glycerol kinase and glycerol-3-phosphate acyltransferase, two of the initial enzymes in phospholipid biosynthesis, were significantly higher in the mycelial form (Table 2). The mycelial form of *C. albicans* has previously been shown to possess lower activities of phospholipid degrading enzymes than did the yeast form (26). Thus, the higher/lower activities of phospholipid biosynthetic/degrading enzymes in the mycelial form of *C. albicans* are consistent with the higher levels of phospholipids in the mycelial form.

Fluorescence polarization studies revealed that the lipids of the mycelial form were more fluid as discontinuities were observed in the fluorescence polarization plots at 36 and 27°C for the yeast and mycelial forms, respectively (Fig. 1). The discontinuity in fluorescence polarization suggests a gel-to-liquid crystalline phase transition. The nature of the fatty acyl chains of phospholipids is a most important factor in determining membrane fluidity,

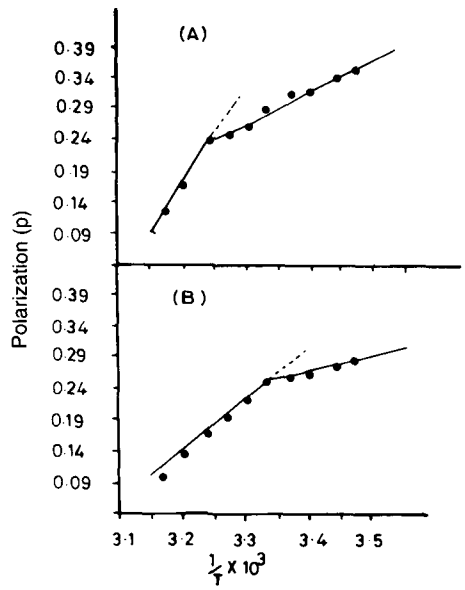

FIG. 1. Fluorescence polarization plots of liposomal membranes prepared from lipids of *Candida albicans*. (A) Yeast form; (B) mycelial form. Fluorescence polarization measurements were carried out as described in the Materials and Methods section.

TABLE 3

Composition of Phospholipid Fatty Acids of Log Phase Cells of Yeast and Mycelial Forms of *Candida albicans*[a]

Fatty acid	(relative percentage)	
	Yeast form	Mycelial form
14:0	40.5	7.4
15:0	13.4	3.6
16:0	16.6	33.9
16:1	12.0	26.8
18:0	2.5	4.8
18:1	11.5	14.4
18:2	3.2	9.2
U/S[b]	0.4	1.0

[a]Values are average of two batches run in duplicate.
[b]Ratio of unsaturated to saturated fatty acids.

which is consistent with the observed increase in the degree of unsaturation in the mycelial form as compared to the yeast form (Table 3). Furthermore, the polarization values decreased with increasing temperature, indicating an increase in probe mobility with increasing temperature. As a decrease in fluorescence polarization is a measure of disorder, the present study suggests mycelial lipids to be more fluid. Membrane fluidity has been reported to play an important role in various cellular functions including permeability (27), regulation of enzyme activity (28) and transport of various nutrients (29).

In addition to the generally accepted role of fatty acids in modulating the fluidity of membranes, the phospholipid head groups, their charge and structure, can also affect fluidity. Previous studies have supported that PE molecules are more closely packed than are PC molecules (30). Because, in the present investigation, the PC/PE levels were found to be high/low in the mycelial form compared to the yeast form of *C. albicans,* a fluorescent probe, ANS, was employed to examine structural changes arising due to modulation of membrane surface charge. Because ANS is not transported into the cells, but remains predominantly bound onto the cell surface, spheroplasts of yeast and mycelial forms were prepared by enzymatically removing the cell wall. Removal of the cell wall did not result in loss of lipids as the lipid content and composition of spheroplasts were the same as those of whole cells (unpublished observation). The analysis of our results from Scatchard plot suggested that the number of phospholipid binding sites was significantly higher in the mycelial form (Table 4), consistent with the presence of higher levels of

TABLE 4

Number of 1-Anilinonaphthalene-8-Sulfonate (ANS) Binding Sites on Spheroplasts Prepared from Log Phase Yeast and Mycelial Form of *Candida albicans*[a]

	Number of ANS binding sites
Yeast form	733 ± 41.63
Mycelial form	1536 ± 32.15[b]

[a]Values are mean ± SD of three independent batches.
[b]$P < 0.01$.

TABLE 5

Km and Vmax Values for Uptake of L-Proline in Log Phase Yeast and Mycelial Forms of *Candida albicans*[a]

	Km (mM)	Vmax (μmol/mg protein/min)
Yeast form	1.25 ± 0.07	0.030 ± 0.003
Mycelial form	2.00 ± 0.20[b]	0.056 ± 0.008[b]

[a]Calculated from Lineweaver-Burk plots. Values are mean ± SD of three different batches.
[b]$P < 0.05$.

TABLE 6

Minimum Inhibitory Concentrations of Antifungal Drugs for the Yeast and Mycelial Forms of *Candida albicans*[a]

	Amphotericin B (μg/mL)	Nystatin (U/mL)	Miconazole (μg/mL)
Yeast form	3.6	27	10
Mycelial form	3.0	16	4.5

[a]Values are average of three different batches.

LPC and PC and lower levels of PE in the mycelial form than in the yeast form.

To explore the effect of altered lipid composition on biochemical functions, the transport of L-[^{3}H]proline was studied in yeast and in mycelial forms. The Km and Vmax values for the uptake of proline were significantly higher for the mycelial form (Table 5), suggesting that the permeases responsible for the uptake of proline are affected to different degrees by the changes in lipid composition in the yeast and mycelial forms. These results agree with the findings of others suggesting that fluctuations in phospholipid and sterol levels can lead to a change in membrane permeability and membrane associated functions, including the activities of membrane bound enzymes (31–33).

To examine the effect of altered lipid composition on the behavior of antifungal drugs, MIC of amphotericin B, nystatin (polyenes) and miconazole (azole antifungal drug) to dimorphic forms of *C. albicans* were examined. The sites of action of polyenes and miconazole are different (34). The mycelial form was found to be more susceptible to these drugs (Table 6), which may be due to the presence of higher levels of phospholipid unsaturated fatty acids (Table 3) and sterols (Table 1) close to the target sites for the polyene antifungal drugs (35,36). Whereas in the case of miconazole the higher susceptibility of the mycelial form may be due to greater accumulation of methylated sterols, miconazole inhibits P-450 dependent 14α-demethylase in the ergosterol biosynthetic pathway (8), leading to accumulation of methylated sterols possibly resulting in the leakage of essential metabolites (37), and ultimately in cell death.

Taken together, the results of this study indicate that the yeast and mycelial forms of *C. albicans* differ in their phospholipid and fatty acid composition, and that the changes in lipid composition are accompanied by changes in structural and functional properties.

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COMMUNICATION

Kinetic Study of Quenching Reactions of Singlet Oxygen and Scavenging Reactions of Free Radicals by α-, β-, γ- and δ-Tocopheramines in Ethanol Solution and Micellar Dispersion

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Quenching reactions of singlet oxygen and scavenging reactions of free radicals by α-, β-, γ- and δ-tocopheramines (Toc-amines) were investigated spectrophotometrically in ethanol and Triton X-100 micellar dispersions. The rate constants of quenching of singlet oxygen (k_Q) by α-, β-, γ- and δ-Toc-amines increased as the total electron donating capacity of the methyl groups at the aromatic ring increased. A plot of logk_Q vs. peak oxidation potential (E_p) was found to be linear and the slope was negative. Similar results were obtained for scavenging of a phenoxyl radical (PhO·). The results suggest that charge transfer plays an important role in these reactions. The k_Q values were found to be 1.30–2.57 times as large as k_Q for α-tocopherol (α-Toc). Although α-Toc has the highest antioxidant activity among natural tocopherols and related phenols, Toc-amines also can serve as antioxidants.

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It is well known that vitamin E (α-, β-, γ- and δ-tocopherol) inhibits the autoxidation of organic molecules in liquid phase. The mechanism involved has been studied extensively by several investigators (1–4). Vitamin E is present in cellular membranes and in edible oils and acts as an antioxidant by protecting polyunsaturated lipids from peroxidation.

The antioxidant properties of tocopherols (Toc) have been ascribed to hydrogen transfer from the OH group in Toc to a peroxyl radical (LOO·). The hydrogen transfer produces a tocopheroxyl radical (Toc·), which combines with another LOO· (Reactions 1 and 2) (5,6).

$$\text{LOO}\cdot + \text{Toc} \xrightarrow{k_1} \text{LOOH} + \text{Toc}\cdot \quad [1]$$

$$\text{LOO}\cdot + \text{Toc}\cdot \rightarrow \text{nonradical products} \quad [2]$$

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Abbreviations: DPBF, 2,5-diphenyl-3,4-benzofuran; EP, 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid; E_p, peak oxidation potential; k_d, rate constant of deactivation of singlet oxygen in pure solvent; k_{obsd}, pseudo-first-order rate constant for reaction between 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl and antioxidant; K_Q, rate constant of quenching of singlet oxygen by antioxidant; k_s, second-order rate constant for reaction between 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl and antioxidant; LOO·, lipid peroxyl radical; PhO·, 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl; SCE, saturated calomel reference electrode; S_s, slope of first-order plots of disappearance of 2,5-diphenyl-3,4-benzofuran in presence of antioxidant; S_0, slope of first-order plots of disappearance of 2,5-diphenyl-3,4-benzofuran in absence of antioxidant; Toc, tocopherol and its analogues; Toc·, tocopheroxyl radical and its analogues; Toc-amine, tocopheramine.

In a previous study (7), we measured the reaction rates, k_s, of α-, β-, γ- and δ-Toc with 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (PhO·) in ethanol (EtOH) solution (Reaction 3) using stopped-flow spectrophotometry. PhO· can be regarded as a model for active oxygen radicals (LOO· and others) in biological systems.

$$\text{PhO}\cdot + \text{Toc} \xrightarrow{k_s} \text{PhOH} + \text{Toc}\cdot \quad [3]$$

The second-order rate constants, k_s, obtained were 5.12×10^3 (α-Toc), 2.24×10^3 (β-Toc), 2.42×10^3 (γ-Toc) and 1.00×10^3 (δ-Toc) $M^{-1}s^{-1}$ in EtOH at 25.0°C. The relative rates (α/β/γ/δ, 100:44:47:20) agreed well with those obtained in studies on the reactivity of Toc toward poly(peroxystyryl)peroxyl radicals (100:41:44:14) using the O_2 consumption method (Reaction 1, Ref. 8). The result suggests that the relative reactivity of Toc in solution probably does not depend on the type of oxyradicals (PhO· and LOO·) used. It appears that both a charge transfer and proton tunneling play an important role in the antioxidant reaction of Toc (9,10).

Lipid peroxidation is also induced by the reaction of a singlet oxygen with unsaturated lipids, and Toc can act as efficient quencher of singlet oxygen (11–13). α-Toc quenches singlet oxygen by both physical quenching and chemical reaction (Reaction 4, Refs. 11–13).

$$^1O_2 + \text{Toc} \xrightarrow{k_Q} \text{physical quenching} + \text{chemical reaction} \quad [4]$$

In reactions of Toc, plots of k_Q vs. k_s show a linear relationship (14).

Tocopheramines (Toc-amines) can also act as antioxidants (15–17). In fact, in the rat red cell hemolysis test Toc-amines show activity equal to or greater than Toc (15). In order to clarify the antioxidant activity of Toc-amine derivatives, we have measured in the present study the rates (k_s) of scavenging of PhO· by α-, β-, γ- and δ-Toc-amines in EtOH solution and in micellar dispersion at 25°C. We have also measured the rate (k_Q) of singlet oxygen quenching at 35°C, and compared the observed rates, k_s and k_Q, with those of α-Toc. The structures of the molecules used in this work are shown in Figure 1.

MATERIALS AND METHODS

α-, β-, γ- and δ-Toc-amines were prepared according to published methods (18). Ethanol, diethyl ether, benzene, cyclohexane, hexane, toluene and methanol were purified by distillation prior to use. Triton X-100 was purchased

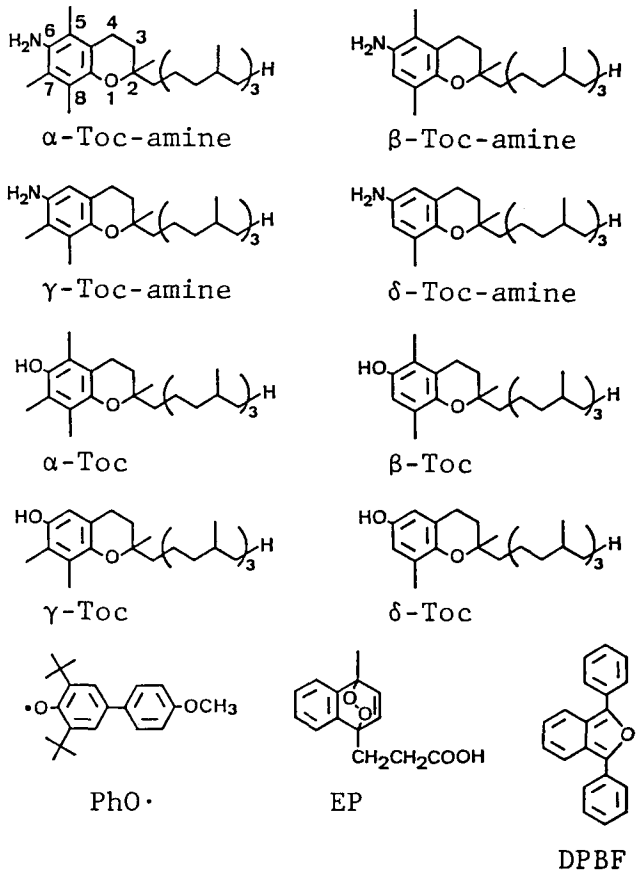

FIG. 1. Structures of molecules used in this work. PhO·, 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl; EP, 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid; DPBF, 2,5-diphenyl-3,4-benzofuran.

from Nacalai Tesque (Kyoto, Japan) and was used as such. The aqueous solution (5.0 wt%) was buffered at pH 7.0 with KH_2PO_4/$NaHPO_4$.

The experimental setup and procedures used for the measurement of k_s have been described in detail previously (19). Briefly, the kinetic data were obtained with a UNISOKU stopped-flow spectrophotometer Model RS-450 (Hirakata, Japan) by mixing equal volumes of solutions or micellar dispersions of a Toc-amine and PhO· under a nitrogen atmosphere. All measurements were made at 25.0 ± 0.5°C. The pseudo-first-order rate constant for the reaction [3] (k_{obsd}) was determined by following the decrease in absorbance of PhO· (375 or 580 nm). The rate constant k_{obsd} is given by Equation 5:

$$k_{obsd} = k_0 + k_s[\text{Toc-amine}] \quad [5]$$

k_0 denotes the rate constant for the natural decay of PhO·; [Toc-amine] refers to the molar concentration of Toc-amine. The rate parameters were obtained by plotting k_{obsd} against [Toc-amine].

The experimental setup and the procedures for the measurement of k_Q have also been described in detail (14). Briefly, the kinetic data were obtained with a Shimadzu UV-2100S spectrophotometer (Kyoto, Japan). Singlet oxygen was generated by the thermal decomposition of 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid (EP; 20,21). 2,5-Diphenyl-3,4-benzofuran (DPBF) was used as standard. The value for k_Q was determined according to Young *et al.* (22). EtOH solutions or micellar dispersions of EP, DPBF and Toc-amine in EtOH were reacted at 35°C. The disappearance of DPBF was measured at 411 nm. The value for k_Q was determined following Equation 6:

$$S_0/S_s = 1 + (k_Q/k_d)[\text{Toc-amine}] \quad [6]$$

where S_0 and S_s denote slopes of the first-order plots of disappearance of DPBF in the absence and presence of Toc-amine, respectively. The value for k_0 can be calculated by using the value of the rate of deactivation of singlet oxygen in pure solvent (k_d), while k_d in EtOH was found to be 8.3×10^4 s^{-1} (23). Since k_d in micellar dispersion is not known, the rate of Reaction 4 in micellar dispersion was estimated to be k_Q/k_d.

The peak oxidation potential (E_p) of Toc-amine was obtained by cyclic voltammetry. Cyclic voltammetry was performed at room temperature under an atmosphere of nitrogen using a Yanaco cyclic voltammetric analyzer Model P-1000H (Kyoto, Japan). The electrodes used were platinum and saturated calomel reference electrodes (SCE) in acetonitrile (dried over P_2O_5) containing 40 mM tetrabutylammonium perchlorate. Under these conditions, ferrocene as a standard sample has a half-wave oxidation potential of +400 mV.

In micellar dispersion, the reactions occur entirely in the hydrophobic phase rather than the bulk phase. Toc-amine, PhO·, EP, DPBF, and singlet oxygen move freely between micelles. Accordingly, the values for k_s and k_Q should be calculated in the micellar volume. However, since the micellar volume is not well known, we calculated the values in the bulk volume. The values for k_s and k_Q thus obtained in the micellar dispersion should be considered relative.

RESULTS AND DISCUSSION

The values of k_s for the reactions of Toc-amine with PhO· in EtOH and micellar dispersion are listed in Table 1 together with E_p and the Toc data. Figure 2 shows plots of $\log k_s$ vs. E_p of Toc-amine and Toc. The k_s of Toc-amine in micellar dispersion and the k_s of Toc in EtOH and micellar dispersion increased as the total electron-donating capacity of the alkyl substituents at its aromatic ring increased and E_p decreased. In Figure 2, the plot for each of these samples indicates a linear relationship. The results suggest that the electron transfer from Toc-amine to LOO· (or PhO·) plays an important role in Reactions 1 and 3 as proposed in the case of Toc (7,9,10); similar to Toc (9,10), both electron transfer and proton tunneling play an important role in the antioxidant reaction of Toc-amine. This electron transfer corresponds to the "normal" region in the Marcus theory (24–26; 27 and references therein) and/or the Rehm–Weller equation (28,29; 30 and references therein).

However, the linear relationship cannot be seen in the reaction of Toc-amine in EtOH; k_s of α-Toc-amine is

TABLE 1

The k_s and k_Q Values for Toc-amine and Toc in EtOH and Micellar Dispersion as Well as E_p[a]

Compounds	k_s[b] ($M^{-1}s^{-1}$) EtOH	k_s[b] ($M^{-1}s^{-1}$) Micellar dispersion	k_Q[b] ($M^{-1}s^{-1}$) EtOH	k_Q/k_d[b] (s^{-1}) Micellar dispersion	E_p[c] vs. SCE (mV)
α-Toc-amine	3.62×10^2	8.76×10^2	5.30×10^8	4.82×10^3	490
β-Toc-amine	8.57×10^2	7.33×10^2	4.25×10^8	5.97×10^3	520
γ-Toc-amine	9.44×10^2	3.97×10^2	3.85×10^8	3.87×10^3	510
δ-Toc-amine	8.35×10^2	1.97×10^2	2.67×10^8	4.16×10^3	570
α-Toc	5.12×10^3[d]	4.53×10^5	2.06×10^8[e]	2.20×10^3	860[f]
β-Toc	2.24×10^3[d]	1.22×10^5	1.53×10^8[e]	1.70×10^3	920[f]
γ-Toc	2.42×10^3[d]	1.19×10^5	1.38×10^8[e]	1.65×10^3	930[f]
δ-Toc	1.00×10^3[d]	1.04×10^4	0.53×10^8[e]	1.38×10^3	990[f]

[a]E_p, peak oxidation potential; k_d, rate constant of deactivation of singlet oxygen in pure solvent; k_Q, rate constant of quenching of singlet oxygen by antioxidant; k_s, second-order rate constant for reaction between 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl and antioxidant; SCE, saturated calomel reference electrode; Toc, tocopherol and its analogues; Toc-amine, tocopheramine. [b]Experimental errors <±5%. [c]Experimental errors <±10 mV. [d]Reference 7. [e]Reference 14. [f]Reference 19.

smaller than the k_s of other Toc-amines. This result is somewhat surprising since the "expected" relative trend is observed in micellar dispersion. It was therefore of interest to make these measurements in other solvents, especially nonprotic ones. The k_s values for the reactions of Toc-amine with PhO· in diethyl ether, benzene, cyclohexane, hexane, toluene, and methanol are listed in Table 2. As in the case of EtOH, k_s for α-Toc-amine is smaller than for the other Toc-amines in diethyl ether, benzene, and hexane. Thus these results are unlikely due to hydrogen bonding and protic solvation and can generally be found in various solvents. At present, we cannot offer an unambiguous explanation, but it is possible that the electron transfer from α-Toc-amine to PhO· in EtOH corresponds to the "inverted region" in the Marcus theory (24–27) and/or the "activationless region" in the Rehm–Weller equation (28–30).

The k_Q values for the reactions of Toc-amine with singlet oxygen in EtOH and micellar dispersion are also listed in Table 1, together with the data for Toc. Figure 3 shows plots of logk_Q vs. E_p of Toc-amine and Toc. The k_Q of Toc-amine and Toc in EtOH and micellar dispersion increased as the total electron-donating capacity of the alkyl substituents at its aromatic ring increased and E_p

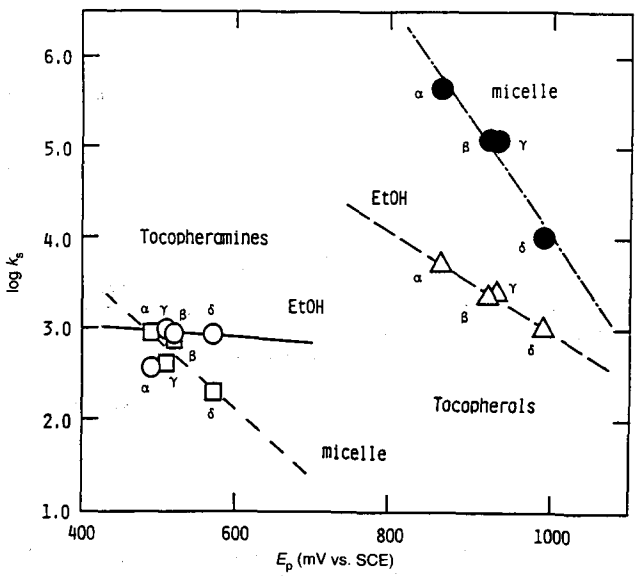

FIG. 2. Plots of logk_s vs. E_p of Toc-amine in EtOH (○), Toc-amine in micellar dispersion (□), Toc in EtOH (△) and Toc in micellar dispersion (●). E_p, peak oxidation potential; k_s, second-order rate constant for reaction between 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl and antioxidant; SCE, saturated calomel reference electrode; Toc, tocopherol and its analogues; Toc-amine, tocopheramine.

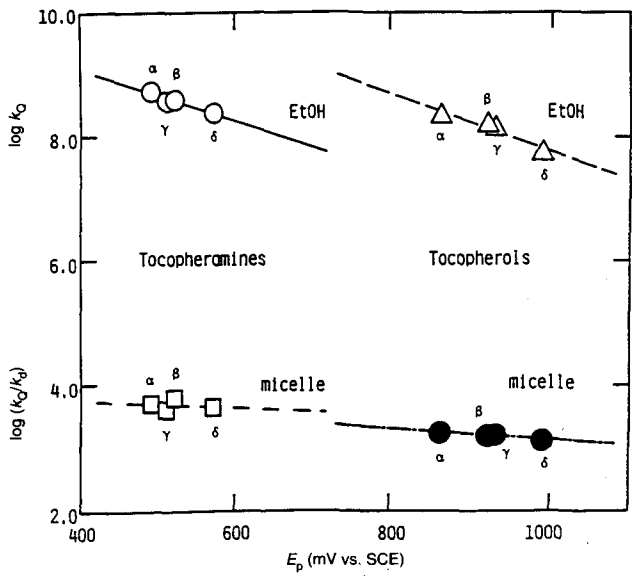

FIG. 3. Plots of logk_Q vs. E_p of Toc-amine in EtOH (○), Toc-amine in micellar dispersion (□), Toc in EtOH (△) and Toc in micellar dispersion (●). E_p, peak oxidation potential; k_d, rate constant of deactivation of singlet oxygen in pure solvent; k_Q, rate constant of quenching of singlet oxygen by antioxidant; SCE, saturated calomel reference electrode; Toc, tocopherol and its analogues; Toc-amine, tocopheramine.

TABLE 2

The k_s Values ($M^{-1}s^{-1}$) for Toc-amine in Various Solvents[a,b]

	α-Toc-amine	β-Toc-amine	γ-Toc-amine	δ-Toc-amine
Diethyl ether	3.85×10^2	6.82×10^2	6.82×10^2	4.06×10^2
Benzene	6.91×10^2	1.03×10^3	1.13×10^3	8.57×10^2
Cyclohexane	8.15×10^2	9.71×10^2	1.14×10^3	6.82×10^2
Hexane	6.04×10^2	8.47×10^2	1.22×10^3	8.63×10^2
Toluene	6.13×10^2	—	—	—
Methanol	6.76×10^2	—	—	—

[a]k_s, second-order rate constant for reaction between 2,6-di-*t*-butyl-4-(4-methoxyphenyl)-phenoxyl and tocopheramine; Toc-amine, tocopheramine.
[b]Experimental errors <±5%.

decreased. In Figure 3, the plot for each of these samples shows a linear relationship, although the slope for Toc-amine in micellar dispersion is very small. The reaction of singlet oxygen with phenols is known to be an electron-transfer reaction (20,31).

It would be worthwhile to compare the relative activity in the rat erythrocyte hemolysis test (15,32) with the relative ratio of both k_s and k_Q of Toc-amines and Toc in EtOH and micellar dispersion. Although the activity of Toc-amine was similar to that of the corresponding Toc in the rat erythrocyte hemolysis test (data not shown), k_s of Toc-amine is much smaller than that of the corresponding Toc. On the other hand, the k_Q values of Toc-amines were found to be 1.30–2.57 times larger than k_Q of α-Toc, which has the largest k_Q among natural Toc and related phenols. Thus, Toc-amines have properties that make them useful as antioxidants.

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ARTICLE

Effects of Site-Directed Mutagenesis on the Serine Residues of Human Lecithin:Cholesterol Acyltransferase

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Lecithin:cholesterol acyltransferase (LCAT) is a serine protease-type enzyme that esterifies cholesterol in human plasma and is activated by apolipoprotein A-I in high-density lipoproteins. LCAT contains 22 serine residues, including Ser181, which is thought to be part of the catalytic site. In order to determine the importance of these serine residues in LCAT, we prepared six LCAT mutants: LCAT (Ser19 → Ala), LCAT (Ser181 → Gly), LCAT (Ser208 → Ala), LCAT (Ser216 → Ala), LCAT (Ser225 → Ala) and LCAT (Ser383 → Ala). We also replaced the adjacent asparagine residues in two additional mutants, LCAT (Ser19 → Ala, Asn20 → Thr) and LCAT (Ser383 → Ala, Asn384 → Thr), in order to ascertain the effect of the serines on *N*-glycosylation. The mutant complementary DNA (cDNA) were subcloned into a eukaryotic expression vector (pSG5) and expressed in COS-6 cells. By polymerase chain reaction analysis, LCAT-specific messenger RNA (mRNA) was found in all mutant and wild-type transfectants. Western blot analysis revealed LCAT-specific bands in media and lysates of the transfected cells. With two exceptions, the amounts of LCAT mass secreted by the transfectants were similar to that of the wild type (mean, 90% mass of wild type; range, 34–138%). Except for LCAT (Ser181 → Gly), which was inactive, the specific activities of the remainder of the mutant enzymes were also similar (mean, 95% activity of wild type; range, 65–169%). These results indicate that Ser181 is part of the catalytic site and that stereoconservative substitutions for serines have minor effects on the synthesis, secretion and specific activities of human LCAT.

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Lecithin:cholesterol acyltransferase (LCAT;EC 2.3.1.43), which catalyzes the transfer of an acyl group from phosphatidylcholine (PC) to the 3β position of cholesterol, is a key enzyme in reverse cholesterol transport (1,2). Protein and DNA sequencing of LCAT (3,4) reveal a 416-residue glycoprotein containing a mixture of β-structure, α-helix and random coil. The hexapeptide Ile 178-Gly179-His180-Ser181-Leu182-Gly183 was first implicated in catalysis because of its reactivity with an active site reagent (5) and its homology with pancreatic and lingual lipases (3,4). A recent report of mutagenesis *in vitro* supported the role of Ser181 in the active site of LCAT (6). Ser216, which appears in a tripeptide sequence (Gly-X-Ser) similar to that of Ser181, is another residue which has been proposed as a part of the active site (7). Moreover, Ser208 and Ser216 are close to the active site region, where small changes in structure might be expected to modify enzyme activity. Thus, it was of interest to determine whether nearby serines play an important role in LCAT activity and to confirm the importance of Ser181 in catalysis. Because serine residues are adjacent to two asparagine residues, Asn20 and Asn384, *N*-glycosylation might be one process that the proximity of serines could affect. A change in glycosylation could, in turn, influence the secretion and activity of LCAT (8,9). In order to assess the importance of the serine residues in LCAT synthesis, secretion and activity, eight mutants were prepared by site-directed mutagenesis, expressed in COS-6 cells, and tested *in vitro*.

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Abbreviations: cDNA, complementary deoxyribonucleic acid; DAPC, 1,2-diarachidonoyl phosphatidylcholine; dATP, deoxyadenosine triphosphate; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; IgG, immunoglobulin G; LCAT, lecithin:cholesterol acyltransferase; mRNA, messenger RNA; PC, phosphatidylcholine; PCR, polymerase chain reaction; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; R-HDL, reassembled high density lipoprotein.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Promega (Madison, WI). The Sequenase Version 2.0 DNA Sequencing Kit was obtained from United States Biochemical (Cleveland, OH). Amersham (Arlington Heights, IL) was the source for [α-^{35}S]deoxyadenosine triphosphate (dATP) (1,000 Ci/mmol), [α-^{32}P]ATP (5,000 Ci/mmol) and Na^{125}I. The rabbit anti-goat immunoglobulin G (IgG) was purchased from BioRad (Hercules, CA), ^{3}H-labeled cholesterol from DuPont (Wilmington, DE) and the phospholipids from Avanti Polar Lipids (Alabaster, AL). A full-length cDNA (complementary DNA) clone of human LCAT (pUCLCAT.10) spliced into the *Eco*RI/*Bam*HI sites of pUC19 was kindly provided by Richard Lawn of Genentech Inc. (South San Francisco, CA) (4). The insert contained 102 nucleotides in the 5′-untranslated region, the entire coding region, and 68 nucleotides in the 3′-untranslated region. Both ends were bound by multiple linkers.

Construction of the mutant clones. The cloning and expression strategies were essentially those of Qu *et al.* (9,10). The oligonucleotides used as the primers for mutagenesis (Table 1) were synthesized on a Cyclone Plus DNA synthesizer (Burlington, MA) and purified by an Oligo-pak oligonucleotide purification column (MiliGen/BioSearch; Burlington, MA). Before use, the oligonucleotides were phosphorylated at the 5′ position by T_4 polynucleotide kinase. After digestion by *Eco*RI and *Bam*HI, the pUCLCAT.10 was subcloned into M13mp18 and M13mp19. Single-stranded DNA containing uracil was prepared by using *Escherichia coli* host strain CJ236 (ung$^-$) to culture the M13mp18 or M13mp19 LCAT clones (11). The single-stranded DNA was used as a template and hybridized to the oligonucleotides, which contained mismatched nucleotides coding for the mutant amino acids. After hybridization, the

TABLE 1

Synthetic Nucleotides Used for Lecithin:Cholesterol Acyltransferase Mutants

Amino acid shift	Primer[a]
Ser19 → Ala	5′-TGT-GTG-GTT-A**GC**-GAG-CTC-AGC-3′
Ser19 → Ala, Asn20 → Thr	5′-CCG-TGT-GTG-G**G**T-A**GC**-GAG-CTC-AGC-3′
Ser181 → Gly	5′-ACA-GCC-GAG-GC**C**-GTG-GCC-AAT-3′
Ser208 → Ala	5′-AGC-CCC-AAG-AG**C**-GAT-GAA-GCC-3′
Ser216 → Ala	5′-GGG-CTT-GAT-GG**C**-GCC-ACC-CCA-3′
Ser225 → Ala	5′-GTT-GTC-ACC-TG**C**-GGC-CAA-GAC-3′
Ser383 → Ala	5′-GGT-CAG-GTT-G**GC**-GAA-GAC-CAT-3′
Ser383 → Ala, Asn384 → Thr	5′-CAG-GGT-CAG-G**G**T-G**GC**-GAA-GAC-CAT-3′
Polymerase chain reaction primers	
Downstream primer	5′-TTC-TGG-CTC-CTC-AAT-GTG-CTC-3′
Upstream primer	5′-CAA-GTG-TAG-ACC-GCC-GAG-GTC-3′

[a]Mutagenesis primers are the antisense strands; the substituted nucleotides are underlined.

second strand was synthesized using T_7DNA polymerase and T_4ligase. The double-stranded DNA was transformed into an ung$^+$ *E. coli* strain, DH-5α′. The plaques of mutant cDNA were identified by the dideoxynucleotide method (12). After digestion by *Eco*RI and *Bam*HI, the wild-type and mutant cDNA were subcloned into a eukaryotic expression vector, pSG5 (13). They were then transformed into *E. coli* AG1. The positive clones were checked by restriction enzyme mapping and direct sequencing. The selected mutant cDNA were prepared using a kit (Qiagen Midi, Chatsworth, CA) to create larger quantities for expression in mammalian cells.

In vitro *expression of LCAT.* A modified diethylaminoethyl (DEAE)–Dextran method (14) was used to transfect wild-type and mutant cDNA of LCAT into COS-6 cells. Cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum and 1× antibiotic–antimycotic liquid (Gibco BRL; Gibco, Gaithersburg, MD) in 75-cm^2 flakes. After exceeding 90% confluence, the cells were subcultured at a 1:4 to 1:6 ratio and incubated for 24–48 h. The cells were transfected with 20 μg of plasmid per flask, incubated for 3 h with chloroquine and the DNA–DEAE–medium stock, and then shocked with 10% dimethylsulfoxide for 2 min. In each experiment, two flasks containing COS-6 cells plus pSG5 plasmids and COS-6 cells only were used as controls. After 72 h of incubation, the media were collected, centrifuged and immediately tested for LCAT activity using model reassembled high density lipoprotein (R-HDL) as the substrate.

RNA preparation, cDNA synthesis and polymerase chain reaction. After the collection of the media for LCAT assays, the transfected COS-6 cells were used to prepare total RNA. Extraction of RNA with guanidinium thiocyanate (15) was followed by centrifugation in cesium trifluoroacetate (16) and precipitation in ethanol. The PCR was performed on the RNA using the GeneAmp RNA PCR Kit (Cetus Perkin-Elmer, Norwalk, CT). PCR was initially conducted for 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. These cycles were followed by 10 additional min of PCR at 72°C and an overnight soak at 4°C.

Quantification of LCAT mass and activity. R-HDL was prepared by a detergent removal technique (17): 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) or 1,2-diarachidonoyl phosphatidylcholine (DAPC), 2 mol% cholesterol and a trace of ^{3}H-labeled cholesterol (7,500 dpm/pmol) were mixed and the organic solvent removed under a stream of N_2. The lipids were vortexed, suspended in tris(hydroxymethyl)aminomethane-buffered saline at pH 7.4, and combined with human apolipoprotein A-I at a lipid protein ratio of 100:1 (M/M). Sodium cholate (10%) was added until the turbidity disappeared. The resulting solution was exhaustively dialyzed against the same buffer, and the LCAT assays were performed as previously described (18). Substrate saturation curves were obtained similarly, except that the substrate concentration was varied as needed.

A solid-phase radioimmunoassay using a goat polyclonal antibody to human LCAT was used to determine LCAT mass; purified human plasma LCAT was the standard. Isolated human LCAT, media from transfected COS-6 cells and the controls (COS-6 cells untransfected or transfected with only the expression vector pSG5) were concentrated tenfold with a Centriprep-30 (Amicon, Beverly, MA). This solution was then applied to a nitrocellulose membrane in a dot-blot apparatus, incubated with antiserum and rabbit anti-goat ^{125}I-labeled IgG, and washed extensively. The dots were cut and the radioactivity was measured by gamma counting.

For the Western blot analysis, electrophoresis was conducted using a 12% sodium dodecylsulfate–polyacrylamide gel, after which the proteins were transferred to a nitrocellulose membrane and reacted with goat antiserum to human LCAT. The bands were visualized using a rabbit anti-goat IgG (heavy and light chains) horseradish peroxidase-conjugated IgG kit (BioRad, Hercules, CA).

RESULTS

Mutagenesis strategy and construction of the expression vector. Alanine and glycine were selected to replace the serines located at residues 19, 181, 208, 216, 225 and 383 of LCAT. These residues were chosen so that the mutated site would retain the same charge. Based on its homology with other lipases (3,4), LCAT's active site has

been assigned to the hexapeptide Ile178-Gly179-His180-Ser181-Leu182-Gly183. We substituted a glycine residue at Ser181 to confirm that it is the likely acceptor of the acyl group transferred from PC to cholesterol during catalysis. Ser208 and Ser216 also contain a portion of the active site structure, Ser-X-Gly, and are close to the putative active site. Moreover, Ser216 reacts with an active site reagent and was once thought to be part of the catalytic site (5). Mutants in which each of these two sites was replaced by alanine were also prepared.

Human LCAT contains four sites of *N*-glycosylation; two of these sites, Asn20 and Asn384, are adjacent to serine residues (3,4). Two mutants, LCAT (Ser19 → Ala) and (Ser383 → Ala), were prepared and tested to determine whether LCAT synthesis, secretion or activity was affected by serine residues vicinal to glycosylation sites. Also, two compound mutants in which an asparagine and the adjacent serine were replaced by threonine and alanine, respectively, were prepared to identify interactions between serines and asparagines that might influence these processes in LCAT. In all, eight cDNA mutants of LCAT were prepared and tested. The oligonucleotide primers used for mutagenesis are listed in Table 1. After mutation, the mutant cDNA were subcloned into a eukaryotic expression vector, pSG5 (11,12,15). The coding regions corresponding to the mutated amino acids were confirmed by DNA sequencing. Sequences of wild-type and mutant LCAT cDNA bracketing the mutation sites are compared in Figure 1. The mutant and wild-type cDNA were expressed in COS-6 cells (11,12).

PCR analysis. Using the primers listed in Table 1, the total RNA of COS-6 cells transfected by pSG5, with and without the inserted LCAT cDNA, were analyzed by PCR. LCAT-specific mRNA (messenger RNA) is indicated by the presence of a 561-bp fragment as a result of agarose gel electrophoresis; this fragment was not found in the cells with pSG5 vector control (Fig. 2). In contrast, the wild-type and all of the mutants contained the 561-bp fragment. Thus, we established that all the cloned cDNA had transcribed their respective mRNA.

Western blot analysis of secreted and intracellular LCAT. Cell lysates from the transfected COS-6 cells were tested by Western blot analysis using a goat anti-

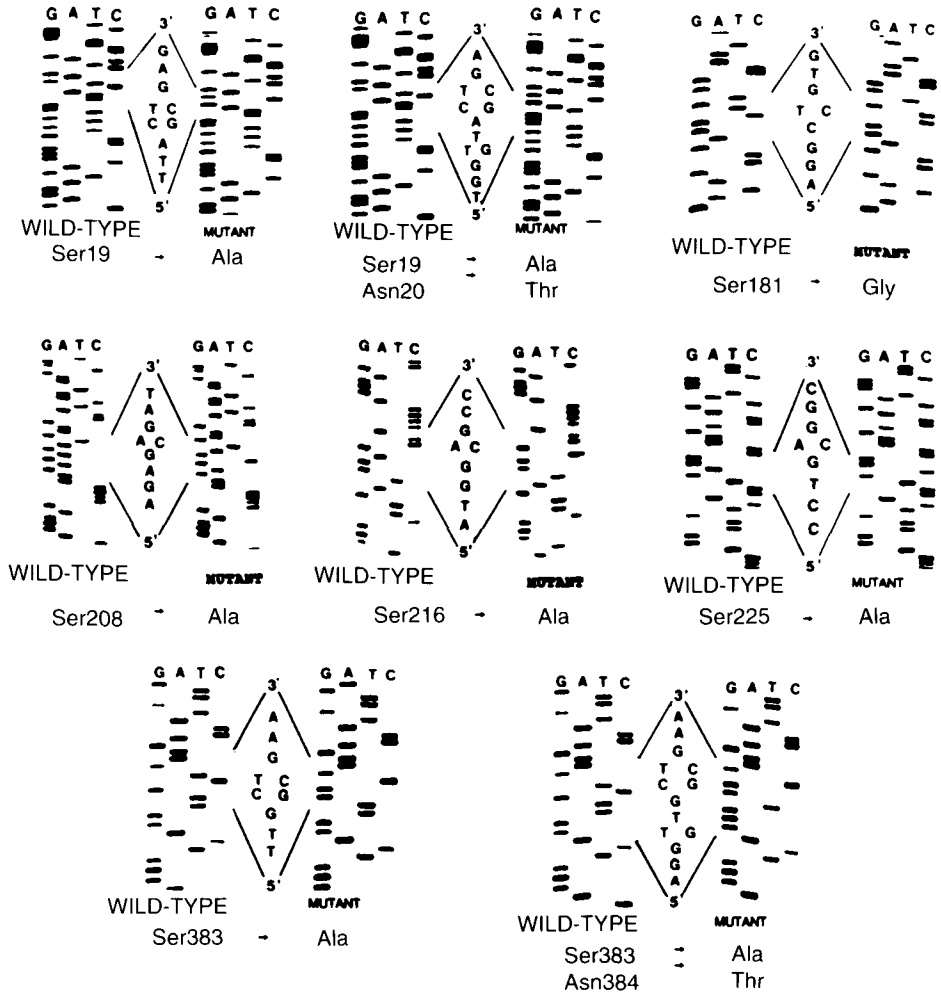

FIG. 1. Comparison of complementary DNA sequences of lecithin:cholesterol acyltransferase (LCAT) from wild-type and mutants. Each pair of autoradiograms contains the sequence of interest of both wild-type LCAT and the site-directed mutant. All sequences are the antisense strand.

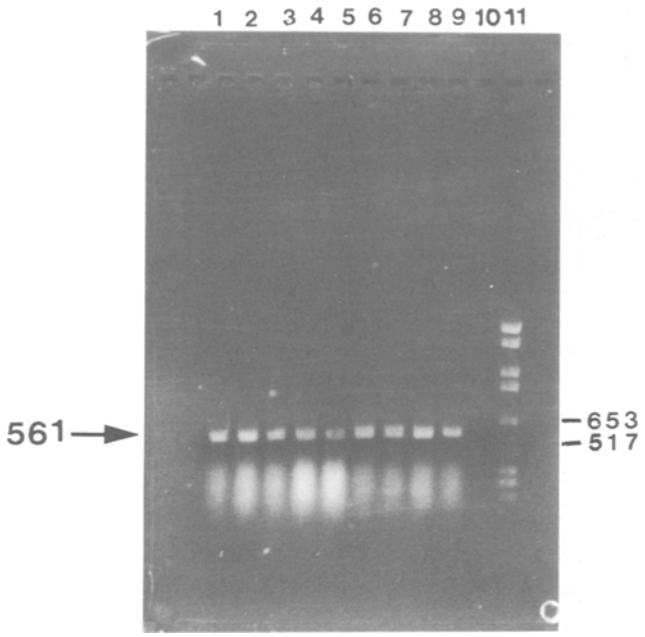

FIG. 2. Polymerase chain reaction amplification of complementary DNA from total RNA. The total RNA sample from COS-cells was subjected to polymerase chain reaction (PCR). Electrophoresis was performed on a 1.2% agarose gel. From the 100 μL of resulting PCR mixture, 5 μL was applied to each lane. Lane assignments were as follows: 1, wild-type LCAT; 2, LCAT (Ser19 → Ala); 3, LCAT (Ser19 → Ala, Asn20 → Thr); 4, LCAT (Ser181 → Gly); 5, LCAT (Ser208 → Ala); 6, LCAT (Ser216 → Ala); 7, LCAT (Ser225 → Ala); 8, LCAT (Ser383 → Ala); 9, LCAT (Ser383 → Ala, Asn384 → Thr); 10, cells transfected by pSG5; 11, marker. Abbreviation as in Figure 1.

human LCAT serum as the first antibody (Fig. 3). Control tests of cell lysates with pre-immune goat serum demonstrated that the additional band corresponding to a molecular weight of about 40kD was due to nonspecific binding. LCAT-reactive bands with molecular weights similar to that of human plasma LCAT were observed when the lysates from cells transfected with the wild-type or mutant cDNA were tested. Lysates from control cells transfected by pSG5 lacking the LCAT insert were negative. These results suggest that all of the cells that contained the mutant and wild-type inserts synthesized LCAT. The lysates from cells transfected with LCAT (Ser19 → Ala, Asn20 → Thr) and LCAT (Ser383 → Ala, Asn 384 → Thr) migrated further than wild-type LCAT. In contrast, no difference was found between LCAT (Ser19 → Ala) and LCAT (Ser383 → Ala) cell-transfected lysate migrations and that of the wild-type LCAT. The media from the transfected cells were also tested by Western blot analysis (Fig. 4). In most cases a single intense band corresponding to the molecular weight of human plasma LCAT was found. However, a number of differences were noted. Although the same volumes of media were applied to the each gel, bands for LCAT (Ser181 → Gly) and (Ser383 → Ala, Asn20 → Thr) were less intense than the others. The bands from the cells transfected with LCAT (Ser19 → Ala, Asn20 → Thr) and LCAT (Ser383 → Ala, Asn384 → Thr) exhibited a greater migration distance, which was consistent with the result that would be given by an LCAT-positive product with a molecular weight lower than that of human plasma LCAT.

Quantification of secreted LCAT mass and activities. Wild-type and mutant cDNA of LCAT cloned into pSG5 were transfected into COS-6 cells. After incubating at 37°C for 72 h, the media were screened for LCAT activity using R-HDL as the substrate. No measurable LCAT activity was found in the media collected from the cells that were not transfected or that were transfected by the pSG5 plasmids lacking the LCAT insert. The media from the COS-6 cells transfected by wild-type and mutant LCAT cDNA exhibited different levels of expression. The media were replaced with DMEM and incu-

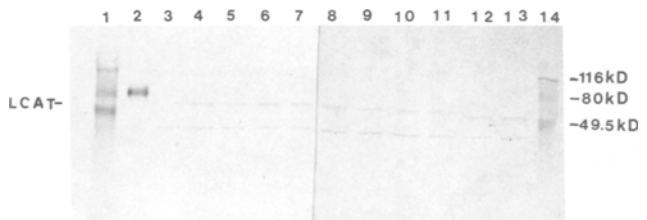

FIG. 3. Western blot analysis of transfected COS-6 cell lysates from control, wild-type and mutants. After incubating for 72 h, cells were washed with phosphate buffered saline and trypsinized. Cells from each 75-cm^2 flask were homogenized using 1 mL of solubilizing buffer (1.6% Triton X-100, 0.3 mM leupeptin, 5.0 M urea and 1.5 mM phenylmethanesulfonyl fluoride). For each sample, 10 μg of protein was used for the analysis. Lane assignments were as follows: 1, marker; 2, standard LCAT; 3, cells transfected by pSG5; 4, wild-type LCAT; 5, LCAT (Ser181 → Gly); 6, LCAT (Ser19 → Ala); 7, LCAT (Ser208 → Ala); 8, wild-type LCAT; 9, LCAT (Ser216 → Ala); 10, LCAT (Ser225 → Ala); 11, LCAT (Ser383 → Ala); 12, LCAT (Ser19 → Ala, Asn20 → Thr); 13, LCAT (Ser383 → Ala, Asn384 → Thr); 14, marker. Abbreviation as in Figure 1.

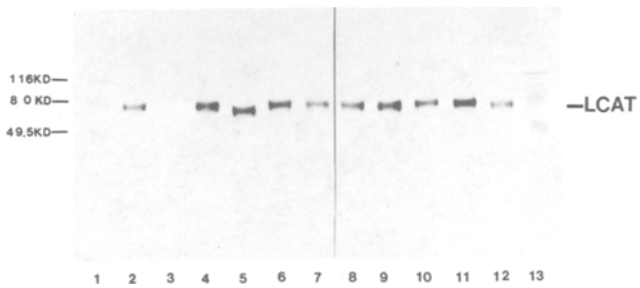

FIG. 4. Western blot analysis of isolated human LCAT, and wild-type or mutant transfected COS-6 cell culture media. The media from COS-6 cells were concentrated tenfold, and 5 μL was applied to each lane. Lane assignments were as follows: 1, marker; 2, standard LCAT; 3, cells transfected by pSG5; 4, wild-type LCAT; 5, LCAT (Ser19 → Ala, Asn20 → Thr); 6, LCAT (Ser19 → Ala); 7, LCAT (Ser181 → Gly); 8, LCAT (Ser208 → Ala); 9, LCAT (Ser216 → Ala); 10, LCAT (Ser225 → Ala); 11, LCAT (Ser383 → Ala); 12, LCAT (Ser383 → Ala, Asn384 → Thr); 13, marker. Abbreviation as in Figure 1.

TABLE 2

Enzyme Activity, Mass and Specific Activity of COS-6 Cell Media Transfected by Wild-Type and Complementary DNA Mutants of Serine[a]

	Activity (nmol cholesteryl ester/mL medium/h)	Mass (μg/mL medium)	Specific activity (nmol cholesteryl ester/μg protein)
Wild-type	6.24 ± 0.21 (1.00)[b]	1.41 ± 0.22 (1.00)	4.46 ± 0.15 (1.00)
Ser19 → Ala	7.82 ± 0.58 (1.20)	1.42 ± 0.12 (1.00)	5.50 ± 0.41 (1.23)
Ser19 → Ala, Asn20 → Thr	4.44 ± 0.51 (0.71)	1.45 ± 0.10 (1.00)	2.92 ± 0.35 (0.65)
Ser181 → Gly	0.03 ± 0.01 (0.005)	0.48 ± 0.01 (0.34)	0.06 ± 0.02 (0.01)
Ser208 → Ala	10.50 ± 0.90 (1.68)	1.40 ± 0.19 (1.00)	7.50 ± 0.64 (1.69)
Ser216 → Ala	13.12 ± 0.44 (2.10)	1.95 ± 0.21 (1.38)	6.73 ± 0.22 (1.51)
Ser225 → Ala	4.29 ± 0.50 (0.83)	1.46 ± 0.25 (1.00)	2.94 ± 0.34 (0.66)
Ser383 → Ala	6.70 ± 0.39 (1.07)	1.50 ± 0.28 (1.00)	4.47 ± 0.26 (1.00)
Ser383 → Ala, Asn384 → Thr	2.91 ± 0.32 (0.46)	0.75 ± 0.01 (0.50)	3.88 ± 0.42 (0.87)

[a]Results from triplicate transfections performed with each plasmid. After 72 h of incubation, the medium was centrifuged in a Beckman J2-21 centrifuge at 1200 rpm for 10 min; 10 μL was then used to estimate lecithin:cholesterol acyltransferase (LCAT) activity. Mass quantification was performed using a dot-blot method. For each well, 4 μg/mL standard LCAT (10, 20, 40, 80 and 100 μL per well) or the sample medium concentrated twofold (20, 40 and 80 μL per well) was applied to nitrocellulose membranes. LCAT was detected by reaction with goat anti-human LCAT serum and ^{125}I-labeled rabbit anti-goat immunoglobulin G.
[b]Values in parentheses indicate the ratio of the mutant value to that of the wild-type LCAT.

bated for another 48 h. LCAT mass was analyzed by a solid-phase immunoassay using a goat anti-LCAT serum raised by purified plasma LCAT as the first antibody and ^{125}I-labeled rabbit anti goat IgG as the second antibody (Table 2). LCAT activity was measured as well.

With the exception of LCAT (Ser181 → Gly), the secreted masses and activities of the various mutant LCAT species were easily detected (Table 2). If LCAT (Ser181 → Gly) is excluded, the total secreted activity from the transfectants was not greatly different from that of the wild-type (mean, 113% of that of the wild-type; range, 46–210%). Secreted LCAT mass was almost identical to that of the wild-type (mean, 90%; range, 34–138%). In contrast, secretion of LCAT (Ser181 → Gly) was only 34% of that of the wild type. Small but significant differences among the other mutants were observed. The secreted masses of LCAT (Ser216 → Ala) and LCAT (Ser383 → Ala, Asn384 → Thr) were 138 and 50%, respectively, of that of the wild-type.

Excluding LCAT (Ser181 → Gly), the specific activities of the mutants were similar to that of the wild-type (mean, 109%; range, 65–169%). The specific activities of two simple mutants which contain a portion of the consensus active site sequence, LCAT (Ser208 → Ala) and LCAT (Ser216 → Ala), were significantly higher than that of the wild-type; LCAT (Ser225 → Ala) had a lower specific activity. Addition of an Asn → Thr point mutation adjacent to existing Ser → Ala substitutions at Ser19 and Ser 383 further reduced the specific activities by 13 and 47%, respectively.

Substrate specificities. The substrate specificities of wild-type and mutant LCAT were compared using the R-HDL prepared from POPC and DAPC. According to the apparent V_{max} obtained with POPC substrates (Table 3), LCAT (Ser208 → Ala) and LCAT (Ser216 → Ala) were approximately 1.6 and 2 times as active as the wild-type. The activities of LCAT (Ser19 → Ala), LCAT (Ser225 → Ala) and LCAT (Ser383 → Ala) were 82, 34 and 68%, respectively, of the activity of the wild-type. LCAT (Ser19 → Ala, Asn20 → Thr) displayed 28% of the activity of the wild-type, while LCAT (Ser383 → Ala, Asn384 → Thr) displayed 43% of the wild-type activity. The R-HDL containing DAPC had a much lower reactivity for all of the mutants, giving results in a pattern similar to that of the substrate containing POPC. The values for the ratio of the apparent V_{max} using POPC to the

TABLE 3

Kinetic Constants for Wild-Type Lecithin:Cholesterol Acyltransferase and Serine Mutants

	POPC		DAPC		
	V_{max}[a]	$K_m \times 10^{8}$[b]	V_{max}	$K_m \times 10^{6}$	R[c]
Wild type	16.0	22.0	2.6	49	6.8
Ser19 → Ala	13.0	19.0	2.0	110	6.7
Ser19 → Ala, Asn20 → Thr	4.4	22.0	0.8	34	7.5
Ser208 → Ala	27.0	22.0	5.4	85	5.0
Ser216 → Ala	35.0	25.0	9.0	110	3.9
Ser225 → Ala	5.6	19.0	0.6	85	9.3
Ser383 → Ala	10.8	23.0	0.8	40	13.5
Ser383 → Ala, Asn384 → Thr	6.8	8.0	0.8	30	8.5

[a]Apparent V_{max} is expressed in nmol cholesteryl esters formed/mL/h.
[b]Apparent K_m is expressed in moles phosphatidylcholine/L.
[c]R is the ratio of V_{max} using 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) to V_{max} using 1,2-diarachidonoyl phosphatidylcholine (DAPC).

V_{max} using DAPC were similar for both the wild type and the mutants.

The affinities of the mutants for the substrate as measured by the apparent K_m were a function of the mutation and the fatty acid composition of the substrate. With POPC as the acyl donor, the apparent K_m values for most of the mutants were close to the value given by the wild-type. The exception was LCAT (Ser383 → Ala, Asn384 → Thr), which had a lower apparent K_m that indicated a higher affinity for the substrate. When DAPC was used as a donor lipid, the affinity of the mutants was similar to or greater than that of the wild-type (mean, 144% of wild type; range, 61–224%). The LCAT mutated at residues 19, 208, 216 and 225 had high apparent K_m values and therefore lower affinity for the substrate. In all cases, consistently lower apparent K_m values demonstrated that the affinity for the substrate was greater with POPC than DAPC.

DISCUSSION

Previous studies of LCAT structure and function, particularly those of fish-eye disease and familial LCAT deficiency (19,20), have shown that a small change in the primary structure involving a single amino acid residue remote from the active site serine can be associated with a large decrease in the rate of secretion and specific activity. In contrast, all but one of the mutations that we prepared resulted in the synthesis of secretion-competent LCAT with a specific activity comparable to that of the wild-type. The LCAT (Ser181 → Gly) mutant was poorly secreted by COS-6 cells and was the only mutant studied that did not catalyze cholesterol esterification. From this result we conclude that this serine is required for catalytic activity. These data provide direct evidence that LCAT is a serine protease-type enzyme and confirm the report of Francone and Fielding (6), who prepared similar mutations in Chinese hamster ovary cells.

A significant enhancement of enzyme activity was observed when Ser208 or Ser216 was replaced by an alanine residue. However, the magnitude of this enhancement was considerably lower than that reported by Francone and Fielding (6), who investigated the effects of mutations at Ser216, but not at Ser208. The segment of LCAT that includes Ser208 and Ser216 is adjacent to the active site region and is not as hydrophobic as the eleven-residue segment containing Ser181 and the five residues on either side. The cause of the higher catalytic activity of LCAT (Ser208 → Ala) and LCAT (Ser216 → Ala) is not known. One possible explanation is that Ser208 and Ser216 may be part of the domain to which a phospholipid molecule binds. Because serine residues are more hydrophilic than alanine residues, the mutation at the serine site may facilitate the hydrophobic binding of substrate molecules. However, a large number of additional mutations would be necessary to make this assignment with certainty.

Two double mutants in which a serine and an adjacent asparagine were replaced by alanine and threonine, respectively, gave rise to secretion-competent proteins. As expected, elimination of the *N*-glycosylation sites (Asn20 and Asn384) was associated with a lower molecular weight due to a lower carbohydrate content. There were no significant differences in the secretion and specific activities of double mutants. We conclude that replacement of serine residues with alanine does not affect the *N*-glycosylation of adjacent asparagine residues and that there is a only a small effect of either mutation or the combination of mutations on LCAT secretion and activity.

The LCAT from different animal species vary considerably in their relative activities against common PC structures, such as POPC, and large, bulky PC molecules, such as DAPC (21–24). This variation has been assigned to possible structural differences, which create steric hindrance to prevent the entry of DAPC into the active site for some molecular species. As with native plasma LCAT, the reactivity of the wild-type enzyme using POPC was about seven times that using DAPC. From this we conclude that selective substitution of alanine residues for serines does not affect the molecular species specificity of human LCAT, and that these residues are likely to be remote from the region of LCAT that binds to PC molecules.

Replacement of serine residues did not greatly change the synthesis, secretion, specific activity or molecular specificity of human plasma LCAT. The one exception was LCAT (Ser181 → Gly), which was poorly secreted and inactive, thereby confirming previous reports that this residue is the active site acceptor of an acyl group from PCs. Thus, our overall conclusion is that the hydrophilic character of serine residues is not essential to the normal functioning of LCAT. Substitution for more than one serine may, however, give different results.

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Cholesteryl Ester Transfer Protein Inhibition by PD 140195

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The presence of plasma cholesteryl ester transfer protein (CETP) activity may be atherogenic, and, therefore, strategies to inhibit its activity or production may result in a beneficial effect on lipoprotein profiles and the disease process. The current report describes 4-phenyl-5-tridecyl-4H-1,2,4-triazole-3-thiol (PD 140195), a novel CETP inhibitor. The concentration-dependent inhibition of CETP by PD 140195 and the inhibitory monoclonal antibody (Mab) TP2 is demonstrated in a variety of *in vitro* assay systems. Molecular models of PD140195 suggest a spatial mimicry of the cholesteryl ester structure. Despite the structural similarity, kinetic studies with a fluorescent cholesteryl ester analog suggest that the inhibition of transfer is not competitive. PD 140195 also selectively inhibited cholesteryl ester but not triglyceride transfer, while the Mab TP2 blocked CETP transfer of both. Studies were carried out to determine whether CETP inhibition observed *in vitro* could also be demonstrated *in vivo*. When PD 140195 was intravenously infused to anesthetized rabbits (up to 20 mg/kg), only transient CETP inhibition was observed. *In vitro* reconstitution studies in the presence of bovine serum albumin resulted in marked reduction of PD 140195 inhibitory activity. Thus, the low activity of PD 140195 in whole plasma probably results from binding to other plasma proteins.

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Cholesteryl ester transfer protein (CETP) is a 74 kDa plasma glycoprotein responsible for the reciprocal exchange of neutral lipids between circulating lipoproteins (1–10). Net alteration in lipoprotein core lipid composition is a complex process. The modifications are influenced by lipoprotein concentration, lipoprotein residence time and the activities of lecithin:cholesterol acyltransferase (LCAT), hepatic lipase, lipoprotein lipase and CETP (11,12). In general, in species lacking CETP activity, and humans genetically-deficient in CETP, the equilibrium favors elevation of antiatherogenic (high density lipoproteins, HDL) and diminution of atherogenic (low density lipoproteins, LDL) lipoproteins (5, 13–22). Therefore, plasma CETP inhibition could be an advantageous pharmacological target for the treatment of dyslipidemic patients at risk for coronary heart disease.

Recent studies of a Japanese family with deficiency in plasma CETP have shown that the deficiency was associated with marked elevation of HDL, its associated apolipoproteins (A-I, E, A-IV) and a rarity of coronary artery disease (13,15–18,23). The defect has been identified as a G to A substitution in the fourteenth intron of CETP pre-messenger RNA (16). This splice donor defect is also the cause of the deficiency in additional Japanese families identified (17,18,20). In a more recent study, the deficiency (both homozygous and heterozygous) has been shown to be associated with a large proportion of Japanese with hyperalphalipoproteinemia (20,24). More recently, a missense mutation at nucleotide 1506 (G for A) has been identified in exon 15 of the CETP gene, resulting in a substution of a glycine for aspartic acid at amino acid 442 (25). The two subjects heterozygous for the missense mutation had three times the normal HDL levels. Overall, these studies suggest that even partial reduction in CETP levels, as found in heterozygous individuals, is associated with elevated HDL. This apparently benign condition (CETP deficiency) has been coined the "longevity syndrome" (13).

A variety of species, which lack CETP activity, including, mice, rats and dogs, have HDL as their major lipoprotein (5). When fed atherogenic diets, transgenic mice expressing human or cynomologus monkey CETP develop atherogenic lipoprotein profiles, including elevation of apolipoprotein B (apoB) containing lipoproteins (very low density lipoproteins, VLDL; and beta migrating cholesteryl ester-enriched very low density lipoproteions, β-VLDL) and reduction of HDL (14,21). These mice also develop atherosclerotic lesions (26). In the transgenic mice, CETP plasma activity has also been shown to be directly correlated with apoB and inversely correlated with apolipoprotein A-I (apoA-I) levels. Infusion of antibodies to CETP into rabbits (27,28) results in a more favorable lipoprotein profile, including elevated HDL cholesterol and particle size. Conversely, infusion of CETP into rats (29,30) results in a less favorable lipoprotein profile, including elevation of VLDL and LDL cholesterol and apoB, and diminution of apolipoprotein E (apoE)-rich HDL cholesterol and HDL size.

Overall, the data suggest that plasma CETP levels contribute to the atherogenicity of plasma. This effect, in part, may be related to the redistribution of core lipids, and alterations in their clearance. Therefore, our focus has been directed to the inhibition of CETP syn-

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Abbreviations: apo, Apolipoprotein; BODIPY-CE, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate; CETP, cholesteryl ester transfer protein; DMA/PEG 200, dimethylacetamide/polyethylene glycol 200; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FCIA, fluorescent cholesteryl ester transfer protein inhibition assay; HDL, high density lipoproteins; HDL_3, high density lipoproteins isolated between the densities of 1.11–1.21 g/mL; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; Mab, monoclonal antibody; PD 140195, 4-phenyl-5-tridecyl-4H-1,2,4-triazole-3-thiol; VLDL, very low density lipoprotein; 1XDB, 50 mM Tris, 150 mM NaCl, 2mM ethylenediaminetetraacetic acid, pH 7.4 buffer; β-VLDL, beta migrating very low density lipoproteins; [^{3}H]CL-HDL_3, high density lipoproteins isolated between the densities of 1.11–1.21 g/mL containing [1,2-^{3}H]cholesteryl linoleate; [^{3}H]TO-HDL_3, high density lipoproteins isolated between the densities of 1.11–1.21 g/mL containing [9,10-^{3}H]triolein.

thesis or activity. The present report describes a novel inhibitor of CETP activity. The activity of this compound on the inhibition of purified and crude CETP preparations is documented. However, when 4-phenyl-5-tridecyl-4H-1,2,4-triazole-3-thiol (PD 140195) was infused in rabbits at high levels only a transient inhibition of CETP activity was observed.

MATERIALS AND METHODS

Preparation of CETP. The d > 1.21 g/mL fraction was isolated from rabbit (Pel-Freez Biologicals, Rogers, AR) or human plasma and dialyzed against 50 mM Tris, 150 mM NaCl, 2mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, buffer (1XDB, pH 7.4). Aliquots were stored frozen at −20°C. Chinese hamster ovary cells transfected with human recombinant CETP (31) were obtained by license agreement (Columbia University, NY). Medium from these cells grown in 10% fetal bovine serum in Hams F-12 was used as a source of human CETP without further purification. The human CETP inhibitory Mab (monoclonal antibody) TP2 was a kind gift from Drs. Ross Milne and Yves Marcel (University of Ottawa Heart Institute, Canada). Mab TP2 is also known to inhibit rabbit CETP (27).

Radioisotopic whole plasma CETP assays. The method for preparation of radiolabeled HDL is an adaptation of the methods previously described by Morton and Zilversmit (32). Briefly, egg phosphatidylcholine in chloroform (5.08 nmol), 100 μCi [1,2-^{3}H]cholesteryl linoleate (1 nmol) in toluene, and butylated hydroxytoluene (0.022 nmol) in chloroform were combined and dried under a stream of N_2, and then lyophilized to remove residual solvent. The dried lipids were resuspended in 2 mL of 50 mM Tris-HCl, 0.01% EDTA, 1 mM dithiothreitol, pH 7.4 by vortexing (10 min), followed by sonication (Cole-Parmer Model 8851) for 2 × 20 min. Fifty mL of a human d > 1.063 g/mL fraction in 1XDB, 5.8 mL of 15 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) in 1XDB, and 1 mL of 0.2 M EDTA containing 2% NaN_3 were added, and the mixture was incubated 16–18 h at 37°C under nitrogen. The HDL_3 fraction (d = 1.11–1.21 g/mL) was isolated following removal of the 1.063–1.11 g/mL fraction by sequential ultracentrifugation. This [1,2-^{3}H]cholesteryl linoleate-HDL_3 fraction ([^{3}H]CL-HDL_3) was dialyzed against 1XDB and used in whole plasma CETP assays. In some preparations, we used 100 μCi of [9,10-^{3}H]triolein for incorporation into HDL_3 ([^{3}H]TO-HDL_3) by essentially the same method used for the incorporation of [1,2-^{3}H]cholesteryl linoleate. Typically, about 25% of [1,2-^{3}H]cholesteryl linoleate and 38% 9,10-[^{3}H]triolein were incorporated into HDL_3 preparations.

Inhibitor screens were performed in 102.5 or 205 μL total volumes in deep 96-well polypropylene plates (1.2 mL capacity/well) or glass tubes, respectively. Compounds (final concentrations up to 100 μM) were added in 2.5 or 5.0 μL dimethylsulfoxide (DMSO) and preincubated for 1 h at 37°C with previously frozen human plasma (25 or 50 μL). [^{3}H]CL-HDL_3 (20,000 or 40,000 dpm) in 75 or 150 μL of 1XDB, pH 8.0, was added and incubated at 37°C. Wells were harvested periodically up to 24 h by the addition of a 1.0 mL solution (per 102.5 μL incubation) containing 10 mg/mL bovine serum albumin, 1.29 mg/mL bovine intestinal mucosa heparin (Sigma Chemical Co., St. Louis, MO) in 0.14 M $MnCl_2 \cdot 4H_20$ in 1XDB pH 8.0 (33). Samples were mixed, and after 10 min centrifuged at 2200 rpm for 30 min at 10°C in an IEC PR-6000 centrifuge (International Equipment Co., Needham Heights, MA) to precipitate apoB-containing lipoproteins. Supernatant aliquots were counted by liquid scintillation spectroscopy to determine radioactivity remaining in HDL_3.

Fluorescent microemulsion assays. Inhibition of CETP activity in rabbit d > 1.21 g/mL or media from Chinese hamster ovary cells secreting recombinant human CETP was assessed in a defined component system by a microemulsion assay utilizing a fluorescent cholesteryl ester analog (BODIPY-CE) containing 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate in place of the normal fatty acid as previously described in detail elsewhere (34). Studies with BODIPY-CE labeled emulsions were done in flat-bottom 96-well plates (Costar Corporation, Cambridge, MA) containing up to 255 μL of sample per well. The 96-well plates were covered and kept in a humidified 37°C incubator in the time intervals between plate scans. BODIPY-CE fluorescence was detected with a Titertek Fluoroskan II 96-well plate reader (Labsystems, Inc., Research Triangle Park, NC) equipped with a 485 and 538 nm band pass filters in the excitation and emission paths, respectively.

For fluorescent assays, maximal initial transfer velocity was first determined with donor BODIPY-CE containing microemulsions (77 pmol BODIPY-CE in 770 pmol total core lipids) and acceptor microemulsions (7.7 nmol core lipids) by titration with recombinant human CETP or rabbit d > 1.21 g/mL fraction in a total volume of 200 μL 1XDB containing 2.5% DMSO. Inhibitor screens were performed with CETP concentrations representing approximately one-half maximal velocity.

To determine albumin effects on sequestration or inactivation of inhibitors, assays also were performed at various bovine serum albumin concentrations with recombinant human CETP, BODIPY-CE containing microemulsions (144 pmol BODIPY-CE) and tenfold excess acceptor microemulsions in a total volume of 250 μL 1XDB containing 2% DMSO.

For other fluorescent CETP inhibitory assays, BODIPY-CE containing microemulsions (0.3, 0.6 or 1.2 nMol BODIPY-CE) were added to 200 μL whole plasma in the absence or presence of inhibitors in a total volume of 255 μL 1XDB containing 2% DMSO. Inhibition data were linear up to 6 h and essentially identical independent of the three different trace amounts of BODIPY-CE containing microemulsion added.

CETP kinetic assay. To assess the inhibition kinetics, we utilized the BODIPY-CE microemulsion assay with increasing amounts of acceptor Intralipid emulsions. Compounds at various concentrations were incubated with variable amounts of Intralipid, a constant amount of BODIPY-CE microemulsion (0.385 nmol BODIPY-CE) and a rabbit CETP source (20 μL plasma or d > 1.21

g/mL). Fluorescence yield was periodically measured to determine initial velocity.

Infusion studies Male Kuiper rabbits (1.7 kg average weight) anesthetized with Rompum (15 mg/kg) and Ketamine · HCl (50 mg/kg) were used to study the *in vivo* effects of PD 140195. Pretreatment blood samples were obtained from a marginal ear vein. PD 140195 (20 mg/mL) solubilized in a dimethylacetamide/polyethylene glycol 200 (DMA/PEG 200) (25:75, vol/vol) vehicle was intravenously infused at 0.1 mL/min to obtain dosages of 10 or 20 mg/kg. Additional blood samples were taken 0.5, 1, 2 and 4 h following initiation of dosing for *ex vivo* CETP activity determinations.

Plasma aliquots (5, 10, 20 and 30 µL) were incubated in a humidified chamber at 37°C with Intralipid (678 nmol triglyceride) and BODIPY-CE microemulsion (1.15 nmol BODIPY-CE in 13.39 nmol total core lipids) in a total incubation volume of 240 µL 1XDB. Fluorescence yields were periodically determined up to 20 h to determine initial transfer rates for *ex vivo* CETP activity under Vmax conditions as previously described (34).

Synthesis of PD 140195. Tetradecanoyl chloride and 4-phenyl-3-thiosemicarbazide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Tetradecanoyl chloride (51 mL, 0.19 mol) was added dropwise at 0°C to a mechanically stirred solution of 4-phenyl-3-thiosemicarbazide ($PhNHCSNHNH_2$; 31.4 g, 0.19 mol) in tetrahydrofuran (1L) and triethylamine (26 mL, 0.19 mol). The solution was allowed to warm to room temperature and stirred for 18 h. The mixture was filtered, the filtrate concentrated *in vacuo,* and then triturated with 50% ethyl acetate/hexane (0.5L) with ice cooling. The resultant precipitate was filtered, washed with 50% ethyl acetate/hexane (2 × 200 mL) and dried for 12 h *in vacuo* to yield tetradecanoic acid, 2-(*N*-phenylaminothioxomethyl)hydrazide (44.4 g) which without further purification was dissolved in a mechanically stirred solution of sodium hydroxide (14.1 g, 0.35 mol) in water (0.5 L). The resultant mixture was allowed to stir until all solid had dissolved, and then for an additional 4 h. The mixture was acidified with 1M HCl (0.5 L) and the precipitate filtered, dried *in vacuo,* and recrystallized from acetonitrile to yield PD 140195 as needles (40.6 g, 59%). Melting point (96–97°C) was determined on a Thomas-Hoover melting point apparatus (Philadelphia, PA) and is uncorrected. ^1H nuclear magnetic resonance (DMSO): δ 13.69 (*s,* 1H), 7.62–7.48 (*m,* 3H), 7.43–7.36 (*m,* 2H), 2.43 (*t,* 3H), 1.50–1.35 (*m,* 2H), 1.28–1.09 (*m,* 20H), 0.85 (*t,* 3H) was obtained on a Bruker AM250 MHz instrument (Billerica, MA). Elemental analyses, ($C_{21}H_{32}N_3S$) C, H, N, S, was determined on a Perkin-Elmer Model 240C elemental analyzer (Madison, NJ), and were within 0.4%.

RESULTS

Random screening of the chemical inventory of Parke-Davis resulted in the identification of several compounds with apparent CETP inhibitory activity. In these initial studies, we incubated whole human plasma with human [^3H]CL-HDL_3 in the presence of DMSO vehicle, CETP inhibitory Mab TP2 (7), or 100 µM compounds for 16 to 24 h. Transfer of cholesteryl linoleate radioactivity to apoB containing lipoproteins was assessed by heparin–manganese precipitation followed by determination of radioactivity remaining in the supernatant (i.e., apoE-free HDL). CETP inhibition by PD 140195 was identified in this manner. Utilizing this whole plasma assay, the time course and concentration dependent CETP inhibition by PD 140195 were determined (Fig. 1). We constructed a molecular model of PD 140195 using the SYBYL molecular modeling system (Tripos Associates, St. Louis, MO) and compared this to the crystal structure of cholesteryl oleate, one of the natural substrates in the transfer process mediated by CETP (Fig. 2). It was interesting to note that PD 140195 could be aligned to mimic either the cholesterol or oleic acid portion of the ester. Both of these alignments position the polar functions of the inhibitor along those of the cholesteryl ester moiety in such a manner as to match the interaction with potential H-bonding recognition sites on the transfer protein. When this is done, PD 140195 was found to mimic almost exactly the length of either the cholesterol moiety or the oleic acid moiety in its extended form. Common putative H-bond donor or acceptor interactions are depicted.

Although the crystal structure of CETP is unknown, the similarities between PD 140195 and cholesteryl

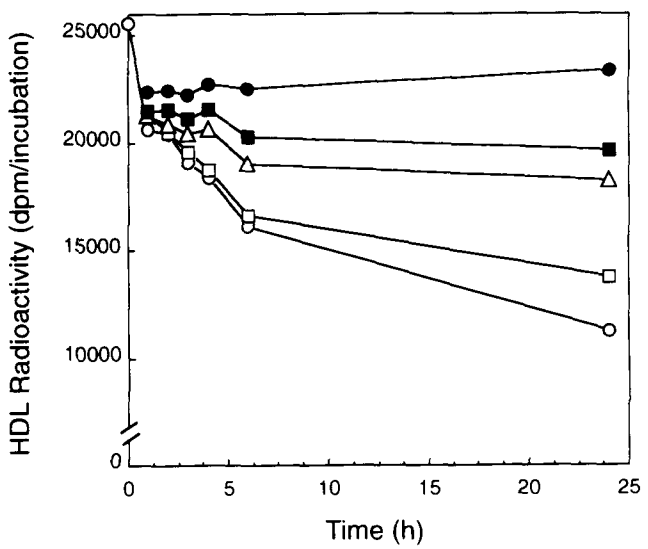

FIG. 1. Inhibition of cholesteryl ester transfer protein (CETP) in whole human plasma by PD 140195. Whole human plasma (50 µL) was incubated with high density lipoproteins (HDL) isolated between the densities of 1.11–1.21 g/mL containing [1,2-^3H]cholesteryl linoleate ([^3H]CL-HDL_3) (40,000 dpm) in the presence of 5 µL dimethylsulfoxide (DMSO) vehicle (○), 5 µg/mL CETP inhibitory monoclonal antibody TP2 (●), or 10 µM (□), 30 µM (△) or 100 µM (■) PD 140195 in a total volume of 205 µL. At intervals up to 24 h, the amount of radioactivity remaining in the supernatant following heparin/manganese precipitation of apolipoprotein (apo) B and apoE containing lipoproteins was determined as described in Materials and Methods. Data represent the average of triplicate incubations for each time point and are representative of a typical experiment.

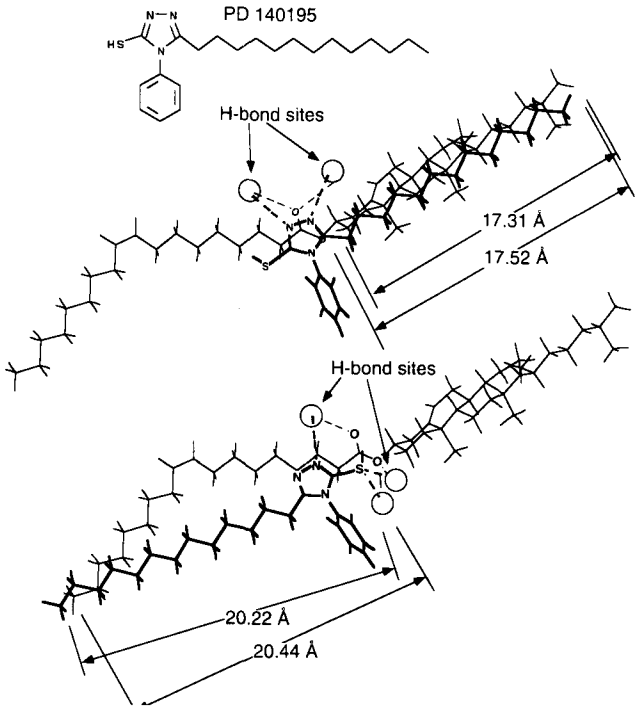

FIG. 2. Structure of PD 140195 and similarity to a cholesteryl ester. PD 140195 (top) is overlayed on cholesteryl oleate in a "side chain-cholesterol mode" (middle) and "side chain-side chain mode" (bottom). Mimicry of potential hydrogen sites for both PD 140195 and cholesteryl oleate are shown for both overlays.

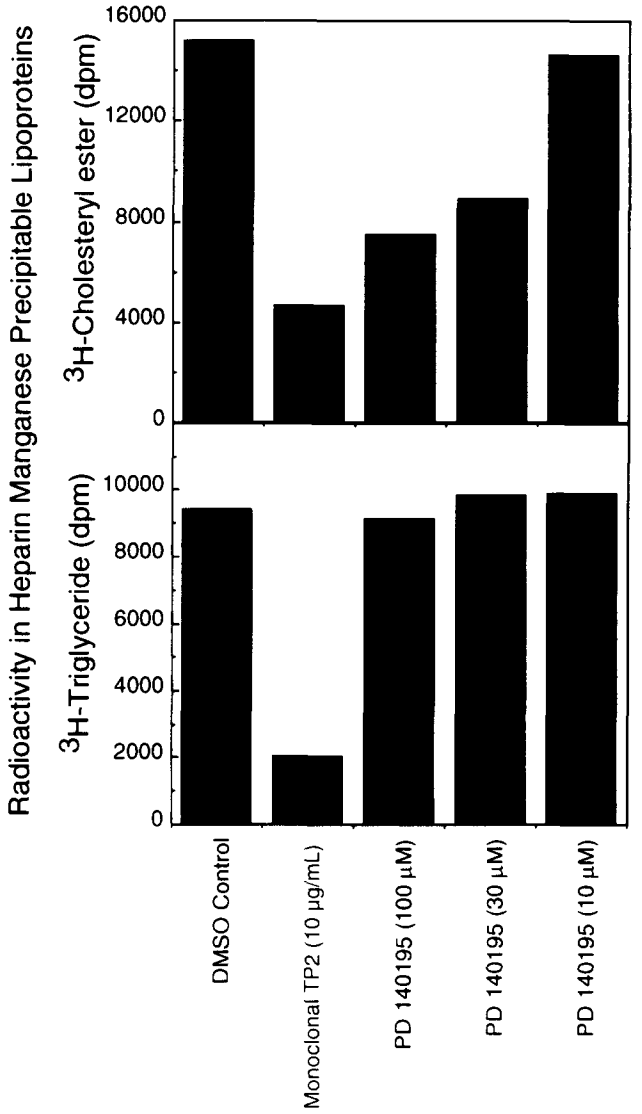

FIG. 3. Effect of monoclonal (Mab) TP2 and PD 140195 on cholesteryl ester transfer protein (CETP) facilitated transfer of cholesteryl oleate and triolein in human plasma. Human plasma (50 µL) was preincubated with Mab TP2 (10 µg/mL), PD 140195 (10–100 µM) or dimethylsulfoxide (DMSO) vehicle (5 µL) for 1 h at 37°C prior to addition of either high density lipoproteins isolated between the densities of 1.11–1.21 g/mL containing [1,2-^{3}H]cholesteryl linoleate ([^{3}H]CL-HDL$_3$) (41,000 dpm) or [9,10-^{3}H]triolein ([^{3}H]TO-HDL$_3$) (45,000 dpm) in a total incubation volume of 205 µL 1XDB. Incubation continued for an additional 17 h at 37°C prior to heparin/manganese precipitation and determination of supernatant radioactivity as described in Materials and Methods. Data show radioactivity transferred to precipitated acceptor lipoproteins (2 to 6 tubes for each condition) and are representative of a typical experiment.

ester suggests transfer inhibition might involve occupation of the cholesteryl ester binding domain. Although triglycerides and cholesteryl esters appear to transfer in an equimolar fashion (35), it is unclear whether a separate or shared domain facilitates this process. CETP site-directed mutations (31) and whole immunoglobulin inhibitory Mab TP2 (36) block both triglyceride and cholesteryl transfer, suggestive of a single site. However, unlike whole immunoglobulin, Fab fragments of Mab TP2 dissociated the transfer rates of cholesteryl ester and triglyceride (36), which is suggestive of independent lipid binding sites. In support of separate lipid binding domains are the studies of Morton and Zilversmit (37) that demonstrated selective inhibition of triglyceride exchange with mercurial compounds. Furthermore, Busch and Harmony (38) demonstrated selective cholesteryl ester inhibition with short-chain cholesteryl esters. Consistent with the data of Swenson *et al.* (36), who utilized [^{3}H]CL-HDL$_3$ or [^{3}H]TO-HDL$_3$ in whole plasma assays, Mab TP2 (whole immunoglobulin) inhibited both cholesteryl ester and triglyceride exchange, while PD 140195 inhibited only cholesteryl ester transfer (Fig. 3).

Although the whole plasma assay was useful in identifying CETP inhibitors, we found utilization of precipitation assays for CETP activity somewhat problematic in that endogenous apoE can rapidly equilibrate to the surface of the added [^{3}H]CL-HDL$_3$ (34,39). This exchange would facilitate [^{3}H]CL-HDL$_3$ precipitation with the apoB containing lipoproteins. In our experience, approximately 10–20% of added HDL radioactivity precipitated with heparin–manganese at zero time. Zero time recovery was further compromised (not shown) when [^{3}H]CL-HDL$_3$ was added to plasma of species containing large amounts of HDL associated apoE (mouse plasma for example). Other assays dependent on physi-

cal separation of donor and acceptor, such as gel filtration (Fig. 3) or ultracentrifugation, are not practical for assessing transfer activity in multiple samples at various time points. Therefore, we developed a fluorescent assay for determination of CETP activity (34). Utilizing this fluorescent CETP inhibition assay (FCIA), the concentration-dependent PD 140195 effects on BODIPY-CE transfer from microemulsions were assessed in whole plasma and component systems and are compared to precipitation assays that utilized [^{3}H]CL-HDL$_3$ as donor in whole human or rabbit plasma (Fig. 4). It should be noted that a significant increase in inhibition was observed in the reconstituted component system that contained recombinant CETP. This apparent difference between reconstituted and whole plasma systems will be addressed.

To assess *in vivo* inhibitory activity, anesthetized rabbits were intravenously infused with PD 140195 in a DMA/PEG 200 vehicle over 10–15 min, resulting in a dosage of 10 or 20 mg/kg. At intervals, blood was obtained for *ex vivo* CETP activity utilizing BODIPY-CE microemulsions under conditions that determine maximal transfer velocity (34). Under these conditions, a transient and concentration-dependent inhibition of CETP was observed, which returned to control levels by 4 h (Fig. 5). In a separate experiment, performed without anesthesia, the duration of the drug effect was considerably shorter (not shown). These observations were somewhat puzzling, in that robust inhibition was observed with the *in vitro* component systems (e.g., Figs. 1 and 3), although, with the various *in vitro* systems, a marked difference (approximately 30-fold) in inhibitory capacity was noted. However, a direct comparison of the *in vitro* systems is not rigorous, since multiple components vary between each system. However, qualitatively, rabbit d > 1.21 g/mL fractions and whole plasmas ap-

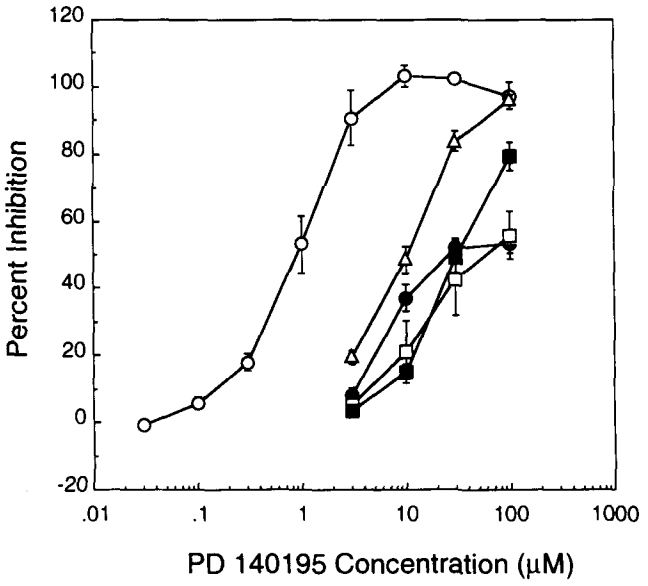

FIG. 4. Inhibition curves for PD 140195 utilizing various assays and cholesteryl ester transfer protein (CETP) sources. Typical PD 140195 inhibitory data utilizing high density lipoproteins (HDL) isolated between the densities of 1.11–1.21 g/mL containing [1,2-^{3}H]cholesteryl linoleate ([^{3}H]CL-HDL$_3$) as the donor lipoprotein in whole plasma CETP assays or cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) containing microemulsions as donor in fluorescent assays. Transfer inhibition of [^{3}H]cholesteryl linoleate from human [^{3}H]CL-HDL$_3$ to human (●) or rabbit (□) plasma heparin-mangenese precipitable lipoproteins was determined after an overnight incubation (16–18 h). Fluorescent assays utilized either recombinant human CETP (○) or rabbit d > 1.21 g/mL fraction (■) as a source of CETP, BODIPY-CE microemulsions as donors, and excess nonfluorescent microemulsions as a BODIPY-CE acceptors as described in Materials and Methods. Fluorescent assays were also performed with BODIPY-CE microemulsions (0.3, 0.6 or 1.2 BODIPY-CE) in 200 μL whole rabbit plasma (△), as a source of CETP and acceptor (endogenous lipoproteins). For fluorescent assays, inhibition was determined at 1–2 h (recombinant human or rabbit d > 1.21 g/mL as CETP source) or 6 h (whole rabbit plasma). Data represent CETP transfer inhibition ± SEM for 3 to 23 experiments at each PD 140195 concentration.

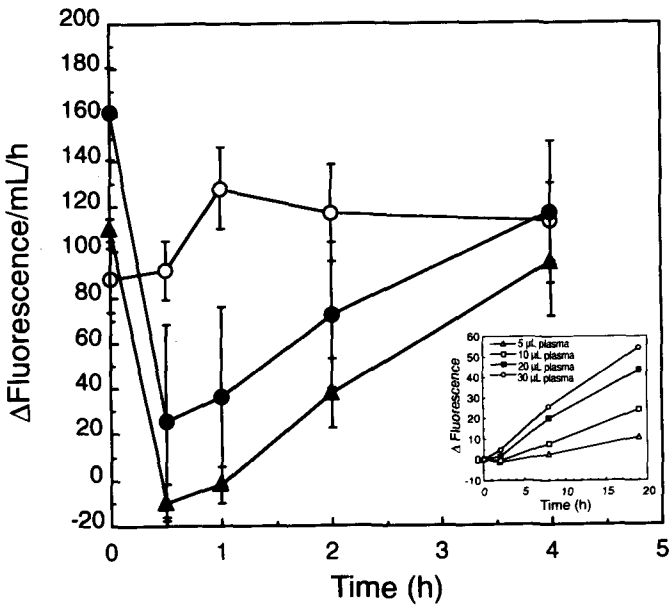

FIG. 5. *Ex vivo* inhibition of cholesteryl ester transfer protein (CETP) facilitated transfer of cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) in microemulsions in plasma from rabbits treated with PD 140195. Anesthetized male Kuiper rabbits were intravenously infused (over approximately 10–15 minutes with a peristaltic pump) with vehicle (○, n = 5), 10 mg/kg PD 140195 (●, n = 4), or 20 mg/kg PD 140195 (▲, n = 4). Blood samples were taken prior to and periodically following the start of infusion. To determine *ex vivo* CETP activity, plasma (5, 10, 20 or 30 μL) from each time point were incubated at 37°C with Intralipid and cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) containing microemulsions in a total volume of 240 μL in microtiter plates. Under these conditions initial transfer velocity is determined under Vmax conditions. Fluorescent yields were periodically determined for up to 21 h (34). A typical assay to determine initial transfer velocity using dilutions of preinfusion plasma is shown (inset). Data shown in large figure represent change in fluorescent yields ± SEM (i.e., *ex vivo* CETP activity in blood samples taken before and up to 4 h following PD 140195 treatment) utilizing duplicate determinations made at 19 or 21 h for 10 μL plasma.

peared to be more refractory to inhibition than recombinant human CETP (Fig. 3). The multiple variables in the whole plasma assays, including the ratio of PD 140195 to CETP and lipoproteins, and that of BODIPY-CE microemulsion levels to lipoproteins, made loss of PD 140195 inhibitory activity difficult to define. These observations suggest that this compound was either competitive with endogenous lipids for CETP or that other plasma components were inactivating PD 140195. The reduced effectiveness of PD 140195 was further investigated in component systems.

To determine if the inhibition was competitive, rabbit plasma or d > 1.21 g/mL was incubated with BODIPY-CE microemulsions with Mab TP2 (2.5–10 μg/mL) or PD 140195 (0.3–3 μM) at various amounts of Intralipid acceptor (Fig. 6). Under these conditions, competitive inhibition was not demonstrable. Possibly, a lack of competition was observed since triglyceride transfer (the major component of Intralipid) is not inhibited by PD 140195. In this regard, we have found that PD 140195 has little or no effect on triglyceride transfer from [^{3}H]TO-HDL$_3$ (Fig. 3). Whether cholesteryl ester emulsions could restore activity in the presence of PD 140195 was not further studied. In a separate assay in which rabbit plasma represented the bulk of the incubation volume, the time course of BODIPY-CE transfer during PD 140195 inhibition was determined and shown to result in a significant decrease in the effectiveness of PD 140195 (Fig. 7). Thus, a component of whole plasma appeared to neutralize this inhibitor.

Next, we investigated the role albumin might play on PD 140195 CETP inhibition. Utilizing, a component system consisting of recombinant CETP, BODIPY-CE microemulsions, and a 10-fold microemulsion acceptor excess in the absence or presence of bovine serum albumin, CETP inhibitory activity was assessed for PD 140195 and Mab TP2 (Fig. 8). Albumin had no inhibitory effect on Mab TP2 inhibition of CETP. In contrast, albumin addition resulted in a concentration-dependent abolition of the inhibitory effects of PD 140195.

DISCUSSION

Whether CETP plays a role in the inhibition or progression of atherosclerosis has been controversial (10). It has

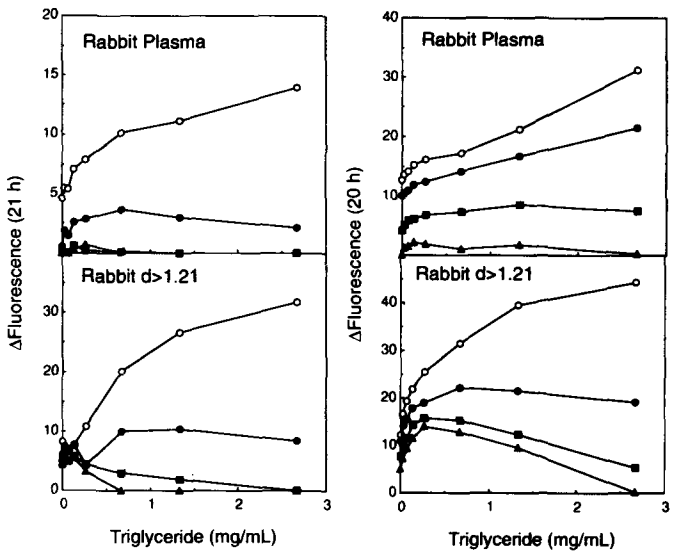

FIG. 6. Effect of triglycerides on PD 140195 inhibition of cholesteryl ester transfer protein (CETP) facilitated transfer. Rabbit plasma (20 μL) or d > 1.21 g/mL (from 20 μL of rabbit plasma) was incubated at 37°C with 2.5 μg/mL (●), 5 μg/mL (■), 10 μg/mL (▲) CETP inhibitory monoclonal antibody (Mab) TP2 (left side graphs), 0.3 μM (●), 1.0 μM (■), 3.0 μM (▲) PD 140195 (right side graphs) or 5 μL dimethylsulfoxide (DMSO) vehicle alone (○) (all graphs) in the presence of a constant amount of cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) containing microemulsion (0.385 nmol BODIPY-CE) and varying amounts of triglyceride emulsion (Intralipid) as indicated in a total incubation volume of 225 μL. Fluorescence was determined at time zero and at 21 h or 20 h, for Mab TP2 and PD 140195, respectively. Data represent the change in fluorescence for duplicate incubations for each condition.

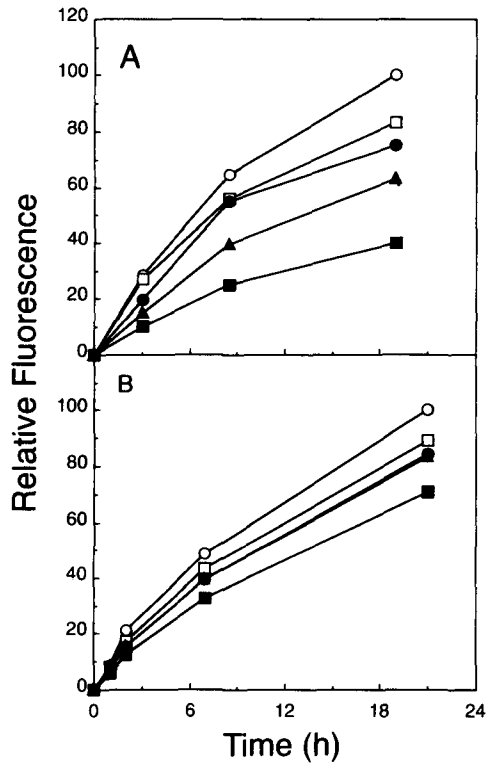

FIG. 7. Cholesteryl ester transfer protein (CETP) activity under conditions in which whole plasma is the predominant assay component. Rabbit plasma (200 μL) was incubated at 37°C with cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) containing microemulsions (20 μL), and 10 μg/mL (●), 20 μg/mL (▲), 40 μg/mL (■) CETP inhibitory monoclonal antibody (Mab) TP2 (panel A), 0.3 μM (●), 3.0 μM (▲), 30 μM (■) PD 140195 (panel B) or 5 μl dimethylsulfoxide (DMSO) vehicle alone (○) in a total volume of 225 μL. Incubations in the absence of DMSO (□) to control for possible nonspecific effects of the vehicle (present with added Mab TP2 and PD 140195) were also performed. Fluorescence was periodically determined up to 21 h. Data are shown as fluorescence change relative to the highest DMSO control values.

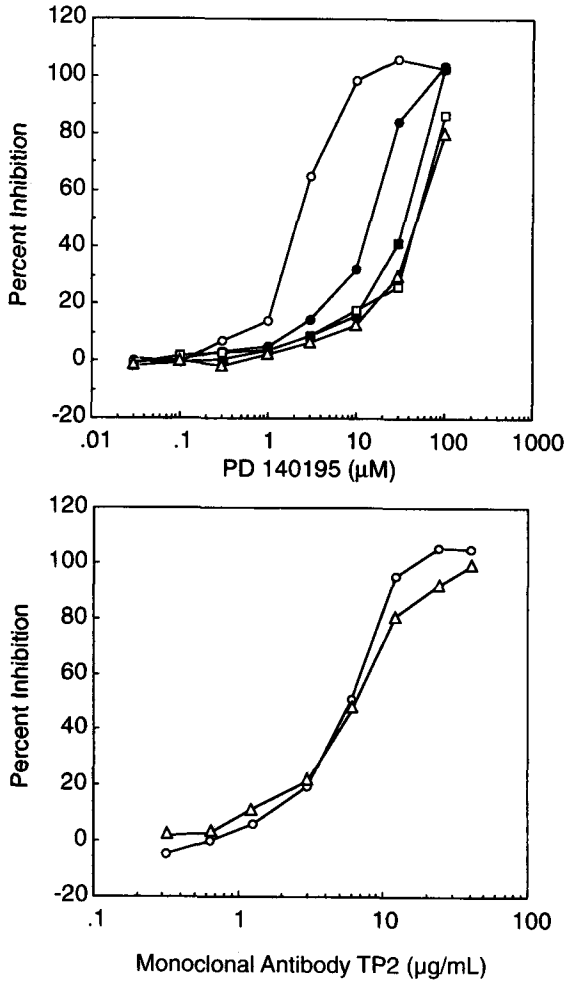

FIG. 8. Effect of albumin on PD 140195 inhibition of cholesteryl ester transfer protein (CETP). PD 140195 (top graph) or monoclonal antibody (Mab) TP2 (bottom graph) was incubated at 37°C with recombinant human CETP (100 μL conditioned media), cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoate (BODIPY-CE) microemulsions (donors), a 10-fold excess of nonfluorescent microemulsion (acceptors) in the absence (○) or presence of 0.5 (●), 1 (■), 2 (□) or 3 (△) percent bovine serum albumin in a total volume of 250 μL. CETP activity was monitored by the loss of fluorescence quenching after 1 h. Data are typical of a represenative experiment.

been hypothesized that CETP plays a central role in reverse cholesterol transport and therefore its inhibition might be atherogenic. However, a direct link between elevated CETP levels and an antiatherosclerotic effect has not been demonstrated. In fact, enhanced delivery of periperial tissue cholesterol to LDL from HDL *via* CETP might reduce hepatic LDL receptor levels and result in an atherosclerotic plasma profile and disease (40). In contrast, CETP reduction appears to be beneficial for inhibiting atherosclerosis progression or enhancing atherosclerosis regression. In humans, homozygous and heterozygous CETP deficiencies are associated with elevated apoE, apoA-I, HDL cholesterol and longevity, and a reduction in LDL cholesterol and coronary heart disease. In general, with the exception of the pig, species that lack CETP activity (i.e., rats, mice and dogs) are resistant to dietary cholesterol induced atherosclerosis; while dietary cholesterol results in atherosclerosis progression in species with CETP activity including CETP-transgenic mice, rabbits, nonhuman primates and humans.

Thus, the inhibition of CETP activity is an intense area of research. Earlier studies have demonstrated that infusion of inhibitory monoclonal or polyclonal CETP antibodies into rabbits results in the appearance of an antiatherosclerotic profile (27,28). More recently, preliminary data have shown that corticosterone administration to CETP-transgenic mice results in decreased plasma CETP levels and reduced hepatic CETP mRNA levels (10). Thus, strategies to inhibit circulating CETP or its synthesis may have beneficial affects on elevating HDL, reducing LDL, and preventing atherosclerosis.

In the current study we describe, in part, our efforts to find simple organic compounds to inhibit circulating CETP. To our knowledge, this is the first report that demonstrates a pharmacologic approach to inhibit CETP. We have described various assays used to monitor CETP activity for the discovery of inhibitors and also have described some of the pitfalls in this discovery process. The major obstacle in this discovery process at this point is movement from an *in vitro* reconstituted system to the whole plasma and to animal systems. Although we found that many inhibitors block CETP activity in reconstituted systems, their specificity to CETP appears to be low in the complex milieu of plasma and in whole animals. In general, the compounds discovered are hydrophobic and might partition into lipoproteins or cellular membranes, thus losing their effectiveness. In addition, binding of PD 140195 and other compounds to other plasma protein components, such as albumin, will also reduce their effectiveness. It should be noted, that albumin (4 g/dL) is in 20,000–40,000-fold molar excess to that of CETP (0.1–0.2 mg/dL) in human plasma. To this end, we have separated inhibitors into those that are and are not (not shown) inactivated by albumin. Some of these compounds not inhibited by albumin are, however, inactive in whole plasma, suggesting that they are bound by other plasma components (Bisgaier, C.L., Essenburg, A.D., Minton, L.L., and White, A., unpublished data). Since antibody studies have demonstrated the feasibility of inhibiting plasma CETP and modulating plasma lipids and lipoproteins, identifying specific CETP inhibitors by novel chemical synthesis will likely result in CETP inhibition in whole plasma and the organism.

Other approaches to CETP inhibition include transcription and translational inhibition. However, much less is known about these processes. Recently, work of Pape *et al.* (41) and Rea *et al.* (42) have suggested that a major source of this protein in nonhuman primates and rabbits is the hepatic nonparenchymal cells, suggesting them as likely targets of pharmacologic intervention. The establishment of stable cell lines to express CETP with its endogenous promoter should enhance pharmacologic strategies for inhibition of CETP production.

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Interactions of MDL 29,311 and Probucol Metabolites with Cholesteryl Esters

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The hypothesis that the efficacy of hydrophobic antioxidants in animal models of atherogenesis may, in part, be related to physical effects on cholesteryl esters in cells was probed with analogs and metabolites of probucol. The interactions of an effective *bis*-thiomethane analog (MDL 29,311) and selected metabolites of probucol with cholesteryl oleate were examined by differential scanning calorimetry and polarized light microscopy. Like probucol, MDL 29,311 and the bisphenol metabolite decrease the liquid-crystalline phase transition enthalpy of cholesteryl oleate with increasing concentrations. At 20 mol%, no transition is detectable. By contrast, the spiroquinone metabolite of probucol and the diphenoquinone metabolite common to both molecules have minimal effects on the liquid-crystalline transitions of cholesteryl oleate. At 20 mol%, neither compound has as great an effect as 1 mol% MDL 29,311. Consistent with their effects on dry cholesteryl oleate, MDL 29,311 and the bisphenol metabolite convert lipid inclusions in cells supplemented with cholesterol to an isotropic physical state similar to that observed with probucol. The number of anisotropic inclusions in the cells decreases with increasing concentration in the medium in the range of 50 to 250 μg/mL. In cells fed with the spiroquinone or diphenoquinone metabolites, the lipid inclusions are liquid-crystalline and resemble those observed with cholesterol-fed controls. These data are interpreted in terms of a model in which hydrophobic antioxidants closely related to probucol disrupt the packing of cellular cholesteryl esters.
***Lipids* 29, 819–823 (1994).**

An early event in the progression of atherosclerosis is the intimal deposition of lipids, including cholesterol and cholesteryl esters (1). This deposition arises from a combination of extracellular and intracellular processes and has been shown in some animal models of atherogenesis to be inhibited by hydrophobic antioxidants such as probucol [*bis* (3, 5-di-*tert*-butyl-4-hydroxyphenylthio) propane] (2). Part of the efficacy of probucol in animal models may be due to modulation of the assembly and molecular interactions of lipoproteins (3). In addition, probucol has been shown to disrupt packing of cholesteryl esters in cells and model lipoproteins on the basis of thermal and microscopic measurements (4). This may contribute to its antioxidant activity in the lipid phase as the rate of oxidation of lipids depends strongly on acyl chain packing (5). The consequence of this fluidizing effect in cells is that the rate of lipid clearance from cholesteryl ester-rich lipid droplets is increased by probucol in the presence of a cholesterol acceptor in the medium (4). These data suggest that hydrophobic antioxidants have both physical effects and antioxidant activities that may be important to the mechanisms by which they inhibit lipid deposition. The present report examines whether such fluidization is common to analogs of probucol that are effective in animal models of cholesterol deposition.

The primary cell types which contribute to lipid deposition in the arterial wall are macrophages and smooth muscle cells. Both cell types accumulate cholesteryl esters to form the foam cells characteristic of atherosclerotic lesions. In cultured cells, cholesteryl ester deposition may be achieved by addition of cholesterol-rich phospholipid liposomes, cholesterol-enriched medium or low density lipoproteins (LDL) that have been modified by acetylation (4,6). Fu5AH rat hepatoma cells are a useful model for studies of the physical properties of the cholesteryl esters deposited in cultured cells. In the presence of cholesterol-enriched medium, Fu5AH hepatoma cells accumulate lipid in an ordered physical state within droplets that exhibit characteristic birefringence cross-patterns when observed through polarized optics. Addition of oleic acid to the medium increases the triglyceride content of the lipid deposits and results in a disordered physical state (6). This increase in fluidity of the lipid droplets increases the rate by which cholesterol may be removed from the cells in the presence of a cholesterol acceptor in the medium.

In LDL receptor-deficient rabbits, an analog of probucol (MDL 29,311) which contains a *bis*-thiomethane rather than the *bis*-thiopropane of probucol (Fig. 1) is effective in reducing aortic surface lesions, but does not significantly reduce plasma cholesterol concentrations (7). The closely related structure of this analog suggested that it may have cholesteryl ester fluidizing activity similar to that of probucol. This activity may be an additional contribution that supplements antioxidant activity in reducing the incidence of fatty lesions in receptor-deficient animals. However, some of the activity of probucol and MDL 29,311 may be the result of their common metabolites. On the basis of studies in monkey liver and adipose tissue (8), probucol has three primary metabolites: the spiroquinone, diphenoquinone and bisphenol (Fig. 1). The present study examines the interactions of MDL 29,311 and the probucol metabolites on cellular cholesteryl esters and the liquid crystalline transitions of cholesteryl oleate.

MATERIALS AND METHODS

Cholesteryl ester loading of cells in culture. The Fu5AH rat hepatoma cell line was graciously provided by J.M. Glick (Philadelphia, PA). Stock cultures were grown in minimal essential medium (MEM) supplemented with basal medium Eagle vitamins, 50 μg/mL of gentamycin,

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Abbreviations: DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; MEM, minimum essential medium.

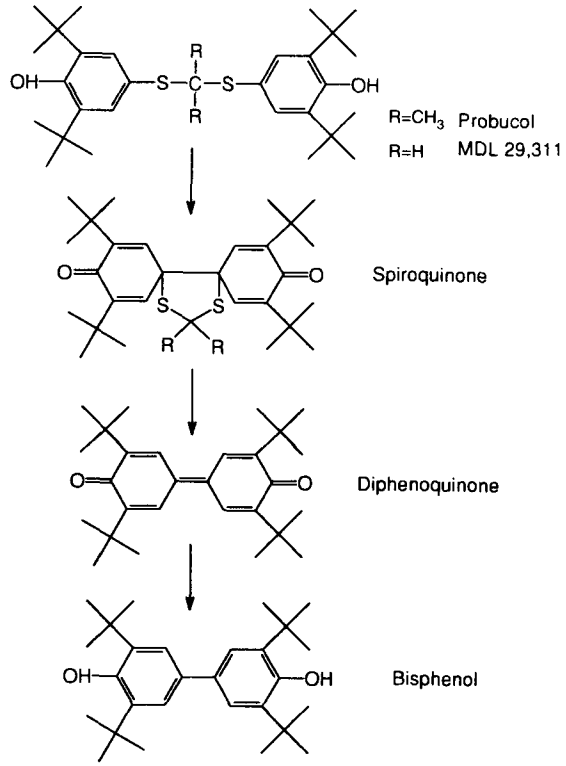

FIG. 1. Structures of probucol, MDL 29,311 and the spiroquinone, diphenoquinone and bisphenol metabolites.

and 5% calf supreme serum (Gibco, Grand Island, NY). Cells were maintained in an atmosphere containing 5% CO_2. Cells were grown in 2-well glass bottom chamber slides (Nunc, Lab-Tek, Naperville, IL) in 2 mL of MEM containing 5% serum for observation of the phase state of lipid inclusions. Loading medium was made by dissolving cholesterol (Calbiochem, La Jolla, CA) in warm (~75°C) absolute ethanol at 10–20 mg/mL and injecting it into warm calf supreme serum while swirling. The mixture was immediately diluted with MEM to a final serum concentration of 10%. Probucol, MDL 29,311, spiroquinone, diphenoquinone and bisphenol were added to serum with cholesterol in a similar manner. The final ethanol concentration was less than 2%. After growth of cells to 70–80% confluence in the chamber slides, the growth medium was replaced with 2 mL of the appropriate loading medium. After loading for 48–72 h, the cells were washed three times with Dulbecco's phosphate buffered saline. Cells were inspected and photographed with a Nikon Microphot-FX microscope. (Melville, NY) equipped with a 10× eye-piece, a 10× objective and polarizing optics.

Differential scanning calorimetry (DSC). DSC scans of dry mixtures of cholesteryl oleate (Nu-Chek-Prep, Elysian, MN) with probucol, MDL 29,311 and the metabolites were made on a Perkin-Elmer DSC-7 (Norwalk, CT). The samples were prepared by mixing in chloroform. A measured aliquot of the solution was transferred to a DSC pan, dried by gentle warming and placed under vacuum overnight. The samples were stored for 10 d at –80°C to promote formation of crystalline material. The samples were scanned at a rate of 2°C/min. Heat capacities and enthalpies were calculated with the software provided by Perkin-Elmer. Transition temperatures correspond to the onset temperatures.

Hydrophobicity estimations. Two high-performance liquid chromatographic (HPLC) systems were used to estimate the hydrophobicity of the molecules. Both employed a C-8 column (5 cm × 4.6 mm id Chromegabond Octyl, 5 μm; Es Industries, Berlin, NJ) and a flow rate of 1.0 mL/min. The dead volume of the column was 0.68 mL. Peaks were monitored at 230 nm with an ABI model 783A detector (Foster City, CA). A Beckman CALS Peak Pro data system (Fullerton, CA) was used for determination of peak retention times and integrations. Mobile phases A and B were mixed by two Waters model 510 pumps (Milford, MA) interfaced with a Waters model 680 controller. In both systems, mobile phase A contained 0.02 M morpholinepropanesulfonic acid and 0.0075 M ethylamine in water adjusted to pH 7.4. In the first system, mobile phase B contained 0.25% octanol in methanol, and a series of isocratic elutions with methanol fractions of 0.98, 0.95, 0.90, 0.85, 0.80 and 0.75 were run. Values in water [log k'(w)] were calculated by extrapolation of the log k' values linearly to zero methanol In the second system, mobile phase B contained 0.25% octanol in tetrahydrofuran, and the sample was eluted with tetrahydrofuran fractions of 0.70, 0.65, 0.60, 0.55, 0.50 and 0.45. An average elution time (midpoint) was calculated for all six runs to give the best estimate of the elution time at 57.5% tetrahydrofuran.

RESULTS

Rat Fu5AH hepatoma cells accumulate lipid droplets that consist primarily of cholesteryl oleate (6). This cell line thus serves as a model system to study the basic features of lipid fluidizing by hydrophobic antioxidants *in vitro*. The propensity of the Fu5AH cell line to deposit lipids in an ordered array when the medium is supplemented with additional cholesterol allows facile manipulation of culture conditions to effect modulation of the physical properties of cellular lipids. Numerous droplets in an ordered anisotropic liquid-crystalline state can be seen in cells observed at room temperature by optical microscopy with crossed polarizers (Fig. 2). The anisotropic liquid crystals appear as bright regions, and the isotropic lipid is dark.

In the experiments shown in Figures 2–4, the cell medium was supplemented with cholesterol (250 μg/mL) and incubated for two days. Probucol, MDL 29,311 and the probucol metabolites were added to the cholesterol-supplemented medium at concentrations of 50, 100 and 250 μg/mL. With the spiroquinone and the diphenoquinone at concentrations up to 250 μg/mL, the cells resemble the cholesterol-fed control cells, with large numbers of birefringent, anisotropic liquid-crystalline droplets (data not shown). With increasing concentrations of probucol, MDL 29,311 or the bisphenol metabolite, a decrease in the number of anisotropic cholesteryl ester droplets was observed at room temperature. At a concentration of 250 μg/mL, the cells contain primarily

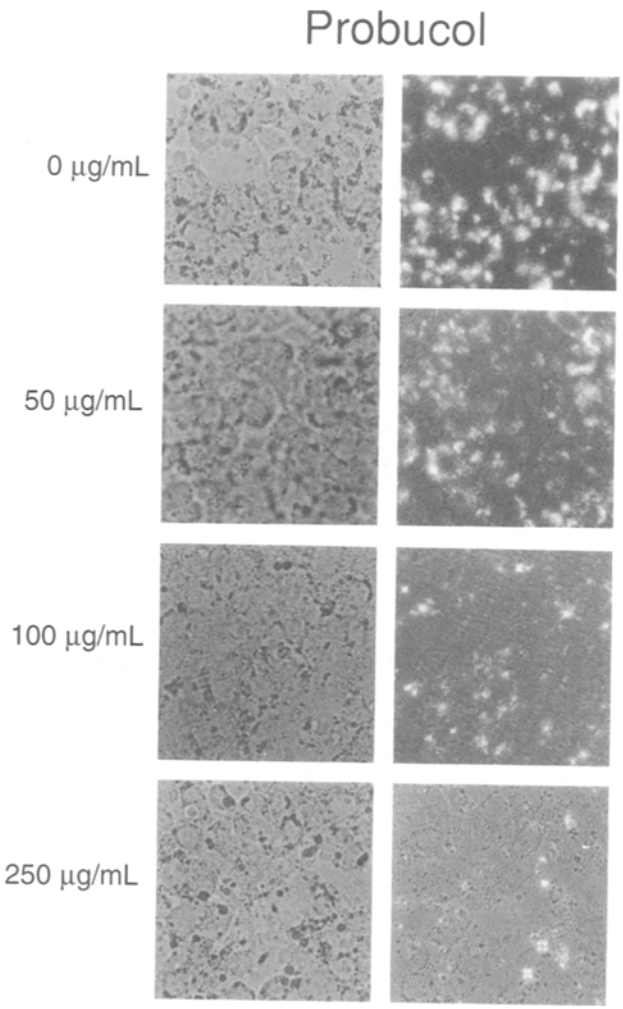

FIG. 2. Light micrographs of living cells fed media enriched with 250 μg/mL cholesterol and varying amounts of probucol observed without (left panels) and with (right panels) crossed polarizers at room temperature.

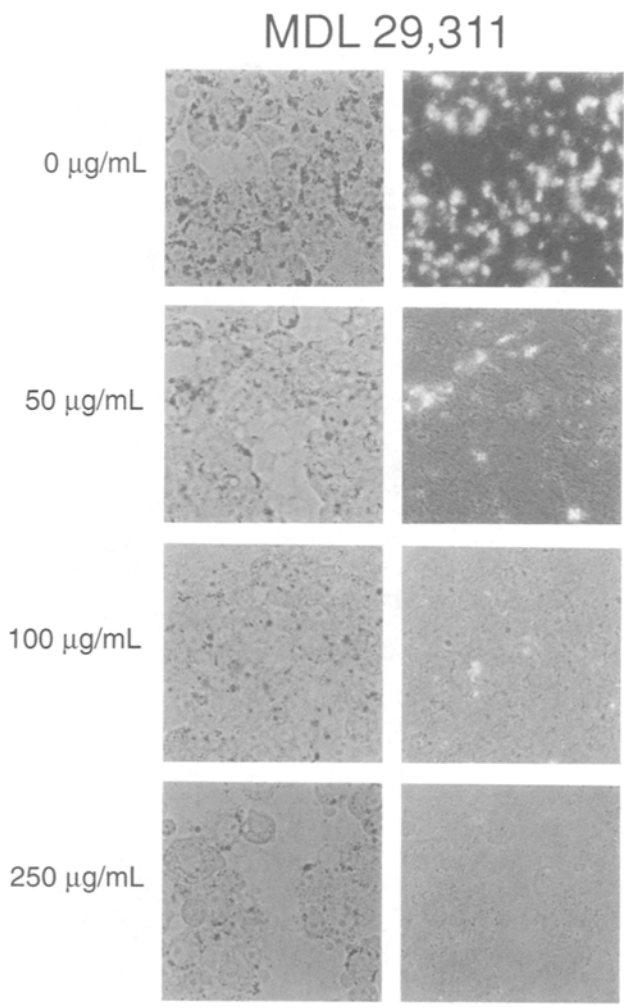

FIG. 3. Light micrographs of cells incubated with cholesterol and increasing amounts of MDL 29,311.

liquid cholesteryl ester droplets which remain dark (isotropic) when observed though crossed polarizers.

To determine whether the effects on cells were due primarily to physical interactions of the analog and metabolites with cholesteryl esters, the effects on dry cholesteryl oleate liquid-crystalline transitions were examined by DSC. This lipid was chosen because of its prevalence in lipid droplets in Fu5AH cells (6). Two sharp phase transitions were observed in pure cholesteryl oleate corresponding to the transformation from isotropic to cholesteric and from cholesteric to smectic phases. The transitions in pure cholesteryl oleate correspond in enthalpy and transition temperatures to those previously reported (4). Due to the overlap of the two transitions, especially in the presence of the analog and the metabolites, only total enthalpies were calculated.

The diphenoquinone metabolite decreased the phase transition temperatures of cholesteryl oleate at concentrations of 20 mol%, with some broadening of the transition and a minimal decrease in the total enthalpy (Table 1). The spiroquinone metabolite broadened the transitions somewhat more at 20 mol%, but the effect was comparable to 1 mol% of the bisphenol metabolite. By contrast, both the bisphenol and MDL 29,311 have a significant effect on the liquid-crystalline phase transitions, even at low concentrations. With this metabolite and the analog at 1 mol%, the cholesteryl oleate liquid-crystalline transitions are significantly broadened, and the phase transition temperatures are depressed by ~4°C. Further broadening of the phase transitions of cholesteryl oleate is observed with increasing concentrations of both MDL 29,311 and the bisphenol metabolite. No discernible phase transition is observed with either compound at concentrations of 20 mol%. The phase behavior and the shapes of the transitions for both MDL 29,311 and the bisphenol metabolite are similar to those previously reported for probucol in the same system (4).

The hydrophobicity of the molecules was examined by HPLC in a series of solvent mixtures on a C-8 column. The retention times of probucol, MDL 29,311 and the bisphenol metabolite are nearly equivalent with methanolic eluants. The calculated log k'(w) values were 10.2 for probucol, 9.4 for MDL 29,311 and 8.9 for the bisphenol metabolite. By contrast, the quinone metabolites are

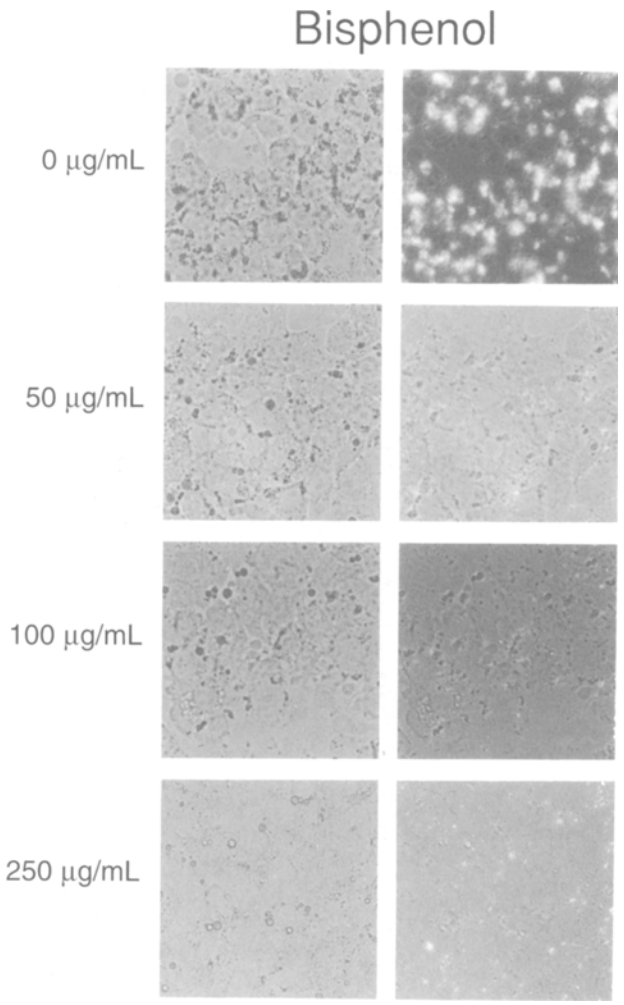

FIG. 4. Light micrographs of cells incubated with cholesterol and increasing amounts of the bisphenol metabolite.

strongly retained on C-8 columns and are not eluted with concentrations <85% methanol. Thus, extrapolation to zero methanol concentration yields misleading results. A second system employing a range of tetrahydrofuran concentrations and calculation of the elution time at 57.5% tetrahydrofuran was also used to give more accurate data for the later-eluting molecules. In this system, the log k′ values relative to probucol were 1.11 for diphenoquinone, 1.04 for the spiroquinone, 0.97 for MDL 29,311 and 0.91 for the bisphenol. These data are consistent with previous HPLC data that indicate that the quinones elute at higher concentrations of organic solvent in gradient systems. (7).

DISCUSSION

Contributions of physical effects to the activities of potentially antiatherogenic compounds have recently been suggested for both probucol (4) and lovastatin (9). Although it is difficult at this point to separate the contributions of physical effects, the data of the present report are consistent with a general phenomenon in which hydrophobic antioxidants fluidize lipids deposited in cells

TABLE 1

Effect of MDL 29,311 and Probucol Metabolites on the Enthalphy and Phase Transition Temperatures of Cholesteryl Oleate

		ΔH (J/g)[a]	T_m (°C)	
Addition	mol%	Total	Smectic	Cholesteric
None		3.4	38.6	44.2
MDL 29,311	1	1.8	34.2	39.5
MDL 29,311	5	0.9	28.9	38.3
MDL 29,311	10	0.3	27.4	
MDL 29,311	20		no transitions	
Bisphenol	1	2.4	36.2	42.0
Bisphenol	5	1.1	31.8	37.2
Bisphenol	10	0.6	27.3	30.9
Bisphenol	20		no transitions	
Spiroquinone	20	3.1	36.2	41.2
Diphenoquinone	20	2.4	33.2	35.5

[a]Standard errors of the enthalpies are ±0.2, and of the transition temperatures are ±0.3

in the presence of excess cholesterol. Vitamin E has a similar effect on both dry cholesteryl oleate and in cells (L.R. McLean, unpublished data). With probucol and related molecules, one must also take care to consider the effects of their metabolic products, which may accumulate to a substantial extent *in vivo*.

The present data show that probucol, a closely related analog, and their common bisphenol metabolite have similar physical effects on cholesteryl ester droplets in cells and in dry mixtures with cholesteryl oleate. In dry cholesteryl esters, this is achieved by decreasing the cooperativity of chain melting during the liquid-crystalline phase transitions. The observed decreases in phase transition temperatures correspond closely to the ~5°C decrease observed with probucol (4). The loss of an observable liquid-crystalline phase transition at concentrations of 20–25 mol% of MDL 29,311 and the bisphenol metabolite is also indistinguishable from the effects of probucol on cholesteryl oleate. These observations in a pure model system are consistent with the effects of the molecules on cellular lipid deposits. By contrast, the diphenoquinone and the spiroquinone metabolites have minimal effects on the phase transition enthalpies of dry cholesteryl esters or the number of anisotropic droplets in cells.

The data are consistent with one of two mechanisms. The first is an alteration in the composition of the intercellular lipid droplets. This possibility has not been ruled out in the present set of experiments. The second is that probucol and the effective analogs act through physical effects on lipid packing. The similarities of the effects of MDL 29,311, probucol and their common bisphenol metabolite on cellular lipid inclusions and on pure cholesteryl oleate are consistent with the latter hypothesis.

Comparison of the structures of the five compounds examined for their effects on cholesteryl ester physical state reveals several important differences. All of the molecules contain two di-*tert*-butyl aromatic rings (Fig. 1). The three active compounds are phenols; the inactive compounds are quinones. The higher hydropho-

bicity of the quinones may explain, in part, their relative lack of efficacy in cell experiments, wherein the molecules may either not readily enter the cells or not be metabolized intracellularly to the bisphenol.

However, it is unlikely that the increased hydrophobicity of the quinones limits their interaction with cholesteryl esters. The quinones, by virtue of the constraints imposed by either the sulfur-containing ring system of the spiroquinone or the double bond of the diphenoquinone, have limited rotational mobility of the aromatic rings and are expected to form a relatively rigid planar system. We propose that the flexibility conferred upon probucol, MDL 29,311 and the bisphenol metabolite by virtue of the unconstrained linkage between their two phenol groups allows rotation of the phenolic rings relative to each other and promotes disruption of the ordered packing of the cholesteryl esters. This hypothesis is also supported by the conformational flexibility that is expected to be retained with a single bond, as is the bisphenol metabolite, which is also active.

The data suggest that physical effects, coupled with the antioxidant activity of MDL 29,311 (7), may contribute to the decrement of fatty lesions in receptor-deficient rabbits without significantly lowering plasma cholesterol levels. One plausible mechanism is that the dynamic turnover of cholesteryl esters in cells is promoted by increasing the fluidity of the lipids. Such a hypothesis is consistent with the proposal that the physical state of the lipids in atherosclerotic plaque is important in determining the rate of turnover of the components of a fatty lesion (10). This fluidizing effect, common to probucol, its bisphenol metabolite and a selected analog, may be an important mechanism of action in reducing arterial fatty lesions in animal models.

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Differential Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Human Skin Fibroblasts

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To better understand the mode of action of ω3 fatty acids in cell membranes, human foreskin fibroblasts were grown in serum-free medium supplemented with 50 μM oleic acid linoleic acid, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), and the effects on membrane composition, fluorescence polarization and enzyme activities were followed. The cells were enriched with EPA and DHA up to 7 and 13% of total lipids, respectively, of which >95% was associated with phospholipids. In addition, the concentration of 22:5n-3 increased with both EPA and DHA to 7.5, and 2.1% of the total fatty acids, respectively. When compared to controls (oleic acid), cells treated with DHA showed a decrease in cholesterol, phospholipids, arachidonic acid (AA) and free cholesterol/phospholipid ratio ($P < 0.05$). In the presence of EPA, only decreases in AA and cholesterol were significant ($P < 0.05$). Membrane fluidity, assessed by fluorescence anisotropy, was increased 16% in cells enriched with DHA ($P < 0.05$), but showed no change with EPA or linoleic acid. There was an increase in membrane-associated 5′-nucleotidase (+27%) and adenylate cyclase (+19%) activities ($P < 0.05$), in DHA-enriched, but not in EPA-enriched cells, when compared with oleate controls. The studies show that incorporation of DHA, but not EPA, into cell membranes of fibroblasts alters membrane biophysical characteristics and function. We suggest that these two major n-3 fatty acids of fish oils have differential effects on cell membranes, and this may be related to the known differences in their physiological effects.

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Epidemiological and clinical studies have shown that ingestion of n-3 polyunsaturated fatty acids (PUFA) is associated with decreased incidence and progression of atherosclerosis-related disorders (1–4). However, the mechanisms by which these n-3 fatty acids exert their beneficial effects are not completely clear.

It is believed that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in marine oils are mostly responsible for the observed beneficial effects (4). As the two n-3 fatty acids are found at varying ratios in different marine oils (5), the relative advantage of one fatty acid over the other has been difficult to assess. Kobatake *et al.* (6) found that EPA selectively lowered triglycerides, whereas DHA selectively lowered cholesterol in rats. Childs *et al.* (7) found that, although all fish oils lowered triglycerides in humans, only DHA-rich tuna and salmon oils reduced low density lipoproteins (LDL) and apoprotein B. Moreover, our laboratory recently reported a differential incorporation of EPA and DHA into plasma lipids (8). We found that relatively more EPA was incorporated into cholesteryl esters, and DHA was preferentially incorporated into phospholipids. These studies suggest that DHA and EPA may be differentially metabolized.

It is well established that the activities of membrane-associated enzymes and receptors can be modulated by changes in membrane lipid composition and in biophysical characteristics (9). It has also been shown that the fatty acids of marine oils are readily incorporated into membrane phospholipids (10). Thus, there has been increased interest in the role n-3 PUFA play in modulating membrane function (11,12). Although several *in vivo* studies have been published on the effects of these marine oils on membrane-associated enzyme activities (13–15), such studies have not been carried out in cultured cells to compare the effects of EPA and DHA on membrane-bound enzymes.

In the present study, we examined the effects of EPA and DHA on the lipid composition and biophysical properties of cell membranes and on the activities of two membrane-associated enzymes, namely adenylate cyclase and 5′-nucleotidase of human skin fibroblasts grown in culture. The results presented here show that EPA and DHA have differential effects on membrane fluidity, as measured by fluorescence polarization, and on the activities of those membrane-bound enzymes.

MATERIALS AND METHODS

Cell culture. Human newborn foreskin fibroblasts (passages 5′–10) were purchased from ViroMed (Minnetonka, MN), and were grown in 75-cm^2 stock flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biologos, Naperville, IL), penicillin (100 U/mL) and streptomycin (100 μg/mL). On Day 0 of the study, confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin 0.02% ethylenediaminetetraacetate (EDTA) solution and were seeded at a concentration of 2×10^5 cells per 75-cm^2 flask containing 10 mL of growth medium supplemented with 10% fetal calf serum. Cells were grown in this medium overnight to allow attachment of cells. The medium was then removed, and the monolayer was washed twice with phosphate buffered saline (PBS). Serum-free medium containing equal volumes of DMEM and F-12 medium, 200 mM glutamine, 2 g/L $NaHCO_3$, 100 μg/mL bovine serum albumin (BSA), 5 μg/mL iron saturated transferrin, 3×10^{-8} M Na_2SeO_3, 15 mM Hepes, 10 ng/mL epidermal growth factor and 5

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Abbreviations: AMP, adenosine 5′-phosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; cAMP, adenosine 3′,5′-monophosphate; DG, diacylglycerol; DHA, docosahexaenoic acid; DMEM, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetate; EPA, eicosapentaenoic acid; GLC, gas–liquid chromatography; LDL, low density lipoproteins; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; TLC, thin-layer chromatography; TMA-DPH, 4′-trimethylammonio-1,6-diphenyl-1,3,5-hexatriene.

μg/mL insulin (Sigma Chemicals, St. Louis, MO) was added along with 50 μM of either oleic acid, linoleic acid, EPA or DHA complexed with human serum albumin (molar ratio fatty acid/albumin, 4:1), together with 20 μM butylated hydroxytoluene (Cayman Chemicals, Ann Arbor, MI). The cells were grown in fatty acid-supplemented medium for four days. Cell numbers and viability were determined by hemocytometer and Trypan Blue exclusion test, respectively.

Fluidity measurements. Cells grown as described above in the presence of various fatty acids were washed with PBS and trypsinized. Approximately 5×10^6 cells were suspended in 1 mL of buffer containing 136 mM NaCl, 11.9 mM $NaHCO_3$, 5.6 mM glucose, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl and 0.42 NaH_2PO_4 (pH 7.4). The fluorescent probe 4′-trimethylammonio-1,6-diphenyl-1,3,5 hexatriene (TMA-DPH) (Sigma) was added to the mixture to give a final concentration of 2 μM. Samples were immediately subjected to polarization analysis at 37°C (excitation wavelength, 366 nm; emission, 450 nm) using a spectrofluorometer with polarization attachment (SLM Instruments, Urbana, IL) (16). The cells were stirred with a magnetic stirrer. Anisotropy (r) was calculated by the equation: $r = 2 \times P/3\text{-}P$, where P is the polarization value. The limiting hindered anisotropy (the static component of steady-state anisotropy) was calculated as: (4 r/3) – 0.1 (17,18).

Lipid analysis. Total cellular lipids were extracted by the method of Bligh and Dyer (19). For gas–liquid chromatography (GLC), the fatty acids were methylated in 1 mL of petroleum ether and 1 mL BF_3 in methanol (14% wt/vol), by heating at 85°C for 15 min and occasional vortexing. The fatty acid methyl esters were dried down under nitrogen, resuspended in hexane for GLC analysis on a Shimadzu GC-9 instrument (Columbia, MD) employing a Supelcowax 10 fused-silica capillary column (8). Authentic fatty acid methyl esters were used for the identification of GLC peaks based on retention times.

For phospholipid analysis, total lipid extracts were applied onto silica gel thin-layer chromatography (TLC) plates (Whatman; American Scientific Products, McGaw Park, IL), and the plates were developed with chloroform/methanol/acetic acid/0.15 M aqueous NaCl (50:25:8:2.5, by vol). This procedure separates lysophosphatidylcholine (R_f 0.1), sphingomyelin (Sph) (R_f 0.2), phosphatidylcholine (PC) (R_f 0.32), phosphatidylinositol plus phosphatidylserine (R_f 0.55), and phosphatidylethanolamine (PE) (R_f 0.8). The phospholipid fractions were visualized by exposure to iodine vapors and scraped from the TLC plate, and lipid phosphorus was estimated by the modified Bartlett procedure (20).

Total cholesterol was measured using an enzymatic kit (Boehringer-Mannheim, Indianapolis, IN) containing cholesterol esterase and cholesterol oxidase. Free cholesterol was measured using a similar assay except that cholesterol esterase was omitted.

Plasma membrane preparation and determination of enzyme activities. Fibroblasts were grown as described above, washed twice with PBS, trypsinized, and centrifuged at $125 \times g$ for 10 min. The cells were washed again, then resuspended in 0.3 M sucrose in a 1:10 dilution of PBS containing 0.15 mM EDTA. The resulting suspension was homogenized and centrifuged according to the procedure of Perdue and Sneider (21) to pellet the plasma membranes.

The assay of 5′-nucleotidase was performed according to the procedure of Lange and Steck (22). Ten μg of cell membrane protein was incubated for 30 min at 37°C in a final volume of 0.75 mL containing 50 mM glycine (pH 9.0), 0.4 mM $MgCl_2$, and 0.16 mM 5′-adenosine monophosphate (AMP). The reaction was stopped by chilling the mixture on ice and adding $ZnSO_4$ and $Ba(OH)_2$ to a final concentration of 37.5 mM each to precipitate unreacted substrate. The mixture was spun at 3000 rpm for 5 min after 10 min on ice. The supernatant was brought to 70 mM $ZnSO_4$ and $Ba(OH)_2$, centrifuged again, and its absorbance was measured at 260 nm. The enzyme activity was expressed as change in absorbance/30 min/mg protein.

Adenylate cyclase activity of the membranes was determined by estimating the formation of 3′,5′ cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) at 37°C (23). One hundred μg of membrane protein was incubated with 0.2 mL of assay solution consisting of 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 250 μM [^{3}H]ATP (ICN Radiochemicals, Irvine, CA), 10 mM creatine phosphate, 0.2 mg/mL creatine kinase, 0.2 mg/mL BSA and 10 mM NaF for 20 min. The reaction was stopped by the addition of 0.1 mL solution of cAMP (50 mM) and ATP (75 mM). The solution was heated at 100°C for 1 min, and after cooling, the cAMP was isolated by the addition of 0.2 mL of 0.25 mM $ZnSO_4$ and 0.2 mL 0.25 mM $Ba(OH)_2$. The radioactivity remaining in the supernatant was counted in a liquid scintillation counter after the addition of 10 mL of scintillation fluid. The enzyme activity was expressed as pmol of cAMP formed/20 min/mg protein.

Protein and DNA determination. The protein content of prepared membranes was determined according to the procedure of Lowry and colleagues (24) using bovine serum albumin (BSA) as a reference standard, after solubilization of membrane preparations in 0.1 N NaOH and 1% sodium dodecylsulfate. The DNA content of cells was determined fluorometrically, using bibenzimidazole dye and calf thymus DNA as a reference standard, according to the procedure of Cesarone *et al.* (25).

Statistics. All results are reported as means ± SEM. The significance of the difference between the means was analyzed with paired and unpaired Student's *t*-test using StatPackets program (Walonick Associates, Minneapolis, MN).

RESULTS

Cellular lipid composition. Table 1 shows that there is no significant difference in cell protein content or DNA content of linoleate-, EPA- or DHA-enriched cells when compared to oleate-enriched controls. No difference in cell growth and viability was observed, as measured by cell counts and Trypan Blue exclusion assay, respectively, upon enrichment with any of the fatty acids (results not shown).

TABLE 1

Cell Viability and Protein and DNA Determination of Fatty Acid-Enriched Cells[a]

Fatty acid enrichment	Protein	DNA	Viability[b] (%)
Oleic	203 ± 30	9.4 ± 1.4	93.7 ± 2.2
Linoleic	201 ± 29	9.3 ± 1.3	93.1 ± 2.0
EPA[c]	211 ± 26	9.8 ± 1.5	92.5 ± 1.9
DHA[c]	208 ± 25	10.0 ± 1.0	93.4 ± 1.6

[a]Values are means ± SEM and are expresed as $\mu g/10^6$ cells. All experiments were done in duplicate (n = 6).
[b]Viability was determined by Trypan Blue exclusion.
[c]EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Cells treated with EPA contained 6.8% of the total fatty acids as EPA, and 7.5% as its elongation product 22:5. The DHA-treated cells contained 13% of total fatty acids as DHA, and 2.1% as 22:5n-3 (Table 2). The increase in the total n-3 fatty acid concentration (20:5 + 22:5 + 22:6) over the oleate control was similar with EPA- and DHA-treated cells (12.1 and 12.9%, respectively). Analysis of the fatty acid composition of total phospholipids and neutral lipids (triglycerides, fatty acids and cholesteryl esters) following their separation on silica gel TLC revealed that over 95% of total EPA or DHA was associated with the phospholipid fraction (results not shown). When compared to oleate-enriched cells, the linoleate-enriched cells showed a significant decrease in 16:0 and 18:1n-7 and an increase in 18:2, 18:1n-7 and 20:3n-6. Both EPA-and DHA-enriched cells showed significant decreases in 16:0, 18:1n-9 and 20:4n-6. The DHA-enriched cells also showed a significant decrease in 18:0. There was some retroconversion of 22:6 to 22:5 and 20:5. However, there was no increase in 22:6 in EPA-treated cells, even though 22:5 was increased. The phospholipid analysis revealed that only DHA enrichment resulted in significant decreases in Sph, PC, and PE (Table 3). The concentration of cholesterol decreased in both EPA- and DHA-treated cells. However, only DHA-enrichment resulted in a significant decrease in the free cholesterol/phospholipid ratio (Table 4). Although the absolute concentrations of all phospholipids decreased in DHA-enriched cells, there was no significant change in the Sph/PC ratio for either EPA- or DHA-enriched cells when compared to controls.

TABLE 2

Fatty Acid (FA) Composition of Total Membrane Lipids (wt%)[a]

Membrane	FA supplemented in the medium			
FA	18:1n-9	18:2n-6	20:5n-3	22:6n-3
14:0	4.0 ± 0.3	3.8 ± 0.3	4.1 ± 0.7	3.8 ± 0.6
16:0	25.7 ± 0.9	18.1 ± 0.9[c]	18.9 ± 0.9[c]	18.3 ± 0.7[c]
16:1n-7	3.6 ± 0.4	4.4 ± 0.7	4.0 ± 0.2	3.7 ± 0.3
18:0	15.2 ± 0.9	15.4 ± 0.6	15.4 ± 0.7	13.4 ± 0.6
18:1n-9	31.1 ± 1.7	22.1 ± 0.8[c]	25.6 ± 1.0[b]	21.7 ± 0.8[c]
18:1n-7	3.1 ± 0.3	4.1 ± 0.7	3.9 ± 0.6	3.7 ± 0.4[b]
18:2n-6	5.6 ± 0.2	15.7 ± 1.3[c]	5.5 ± 0.7	5.5 ± 0.7
20:3n-6	nd	4.2 ± 0.9[c]	nd	nd
20:4n-6	9.5 ± 0.8	9.6 ± 0.6	7.6 ± 0.7[b]	7.1 ± 0.6[b]
20:5n-3	nd	nd	6.8 ± 0.4[c]	2.2 ± 0.3[b]
22:5n-3	nd	nd	7.5 ± 0.7[c]	2.1 ± 0.2[b]
22:6n-3	4.1 ± 0.5	4.6 ± 0.3	2.4 ± 0.2[b]	13.2 ± 0.9[c]

[a]Values are means ± SEM; each experiment (n = 7) was analyzed in duplicate; nd, not detected.
[b]$P < 0.05$, compared to 18:1 control.
[c]$P < 0.001$, compared to 18:1 control.

TABLE 3

Phospholipid Analysis of Fatty Acid-Enriched Membranes[a]

Fatty acid enrichment	Sphingomyelin	PC	PE	PI + PS
Oleic	44.8 ± 2.0	164.9 ± 8.1	103.5 ± 3.4	46.1 ± 1.6
Linoleic	43.1 ± 1.7	161.3 ± 4.7	95.2 ± 3.0	46.5 ± 1.7
EPA	45.1 ± 1.4	165.3 ± 5.3	103.9 ± 3.7	46.4 ± 1.6
DHA	40.1 ± 1.6[b]	143.3 ± 4.0[b]	90.0 ± 3.5[b]	44.1 ± 3.3

[a]Values are means ± SEM, expressed as nmol/mg cell protein. All experiments were done in duplicate (n = 7). PI, phosphatidylinositol; PS, phosphatidylserine. The amount of lysoPC present was negligible in all cells. Other abbreviations as in Table 1.
[b]$P < 0.05$ compared to oleate controls.

Membrane fluidity. The fluidity of fatty acid-enriched plasma membranes was assessed by measuring the rotational mobility of TMA-DPH. As this probe does not enter the cells, it is believed to be specific for the outer leaflet of the plasma membrane of intact cells (16). Our results show that the fluorescence anisotropy as well as its static component, the limiting hindered anisotropy (17), of DHA-enriched membranes were significantly decreased when compared to oleate controls (Table 5). There were no significant changes in the linoleate- or EPA-treated cells.

Adenylate cyclase and 5′-nucleotidase activities. Adenylate cyclase activity was determined by the formation of cAMP from ATP after stimulation with fluoride, which specifically stimulates the regulatory protein subunit (22). Results presented in Table 6 show that there was no significant change in the basal activity upon linoleate, EPA or DHA enrichment. However, the NaF-stimulated activity was increased 20% upon DHA enrichment when compared to controls, but not with linoleate or EPA enrichment.

The activity of 5′-nucleotidase of plasma membranes was assayed by measuring the generation of adenosine from 5′-AMP. We observed a 27% increase in the activity of this enzyme in DHA-treated cells as compared to

TABLE 4

Cholesterol Levels (free cholesterol) and Lipid Ratios of Fatty Acid-Enriched Cells[a]

Fatty acid enrichment	FC	FC/PL	Sph/PC
Oleic	20.4 ± 1.1	0.40 ± 0.03	0.27 ± 0.02
Linoleic	19.4 ± 0.9	0.39 ± 0.05	0.26 ± 0.02
EPA	19.0 ± 1.0[b]	0.37 ± 0.04	0.27 ± 0.01
DHA	15.6 ± 0.8[c]	0.34 ± 0.03[b]	0.28 ± 0.02

[a]All values are means ± SEM. Free cholesterol (FC) values are expressed as μg/mg cell protein. Ratios are expressed as mol/mol. Experiments (n = 6) were done in duplicates. PL, phospholipids; Sph, sphingomyelin; PC, phosphatidylcholine.
[b]$P < 0.05$, compared to oleate controls.
[c]$P < 0.001$ compared to oleate controls.

TABLE 5

Fluorescence Anisotropy of Fatty Acid-Enriched Membranes at 37°C[a]

Fatty acid enrichment	Anisotropy (r)	Limiting hindered anisotropy
Oleic	0.244 ± 0.013	0.219 ± 0.008
Linoleic	0.240 ± 0.012	0.220 ± 0.008
EPA	0.238 ± 0.015	0.218 ± 0.010
DHA	0.210 ± 0.012[b]	0.184 ± 0.009[b]

[a]Anisotropy (r) was calculated following the equation: $r = 2 \times P/3-P$; where P is the polarization value. Limiting hindered anisotropy was determined by the equation: $r = (4r/3) - 0.1$. Values represent means ± SE of six experiments done in duplicates (at least six measurements of each). Abbreviations as in Table 1.
[b]$P < 0.05$ compared to oleate controls.

TABLE 6

Adenylate Cyclase and 5′-Nucleotidase Activities of Fatty Acid-Enriched Membranes[a]

Fatty acid enrichment	Adenylate cyclase		5′-Nucleotidase
	Basal	+NaF	
Oleic	94 ± 21	638 ± 84	64 ± 13
Linoleic	88 ± 21	600 ± 92	70 ± 12
EPA	86 ± 19	672 ± 109	73 ± 14
DHA	90 ± 22	780 ± 94[b]	86 ± 11[b]

[a]Values are means ± SEM for nine experiments done in duplicate. Adenylate cyclase activity is expressed as pmol adenosine 3′,5′-monophosphate formed/20 min/mg protein. 5′-Nucleotidase activity is expressed as change in absorbance units at 260 nm/30 min/mg protein ($\times 10^2$). Abbreviations as in Table 1.

oleate controls (Table 6). No significant change was observed upon linoleate or EPA enrichment.

DISCUSSION

Although both EPA and DHA belong to the n-3 family of fatty acids, and are interconverted in mammalian systems, they exhibit significantly different physical characteristics *in vitro*. Although its "helical structure" allows DHA to behave more like a saturated fatty acid, DHA is still more fluidizing than EPA (26). Studies have also shown that these fatty acids are differentially incorporated into various tissues (9). Taken together, it thus also seemed possible that they have different metabolic effects at the cellular level.

The results presented here show that EPA and DHA do, indeed, have differential effects on membrane structure and function in human skin fibroblasts. Analysis of the fatty acid composition of both EPA- and DHA-enriched cells revealed a significant decrease in arachidonic acid (20:4) content. This is in agreement with previous studies (27) and supports the view that the n-3 PUFA either compete with 20:4 for the *sn*-2 position of membrane phospholipids or inhibit the formation of 20:4 from linoleic acid (18:2) (27). As we found no significant decrease in 18:2 upon either EPA or DHA enrichment, it appears that both mechanisms are active in fibroblasts. Treatment of cells with EPA resulted in a 7.5% enrichment of the membrane with 22:5, suggesting elongase activity in our system.

Only DHA enrichment resulted in a significant decrease in Sph, PE and PC. Because there was no difference in the growth of DHA-treated cells when compared to oleate- or linoleate-enriched cells, it appears that DHA specifically inhibited phospholipid synthesis. It is well established that ω3 fatty acids inhibit cytidinetriphosphate:phosphocholine cytidylyltransferase (28) and phosphatidate phosphohydrolase (29), two key enzymes in phospholipid synthesis. Thus, it is likely that this mechanism was active upon DHA-, but not EPA enrichment.

Cells enriched with DHA showed a significant increase in membrane fluidity as measured by TMA-DPH anisotropy. Other indicators of membrane fluidity were also altered upon DHA enrichment. These include decreases in membrane cholesterol and 18:0 levels and a reduction in the free cholesterol/phospholipid ratio. Although we observed a significant decrease in the cholesterol content of EPA-enriched cells, there was no change in their membrane fluidity. This may have several reasons. First, the membranes were enriched with EPA only up to 6.8% of total membrane fatty acids, compared with 13% for DHA observed upon DHA treatment. Second, there was no significant decrease in 18:0 concentration or in the free cholesterol/phospholipid ratio upon EPA enrichment. Finally, the elongation product of EPA, docosapentaenoic acid (22:5), which comprised 7.5% of the total fatty acids, confers relative rigidity to the membrane when compared to either 20:5 or 22:6 (26). Our observation of no change in membrane fluidity upon linoleate enrichment is at variance with some studies (30) and in agreement with others (31). Possible reasons for the disparate findings include differences in cell types, extent of incorporation of linoleate, and the techniques employed to determine membrane fluidity. Computer modelling of various diacylglycerols (DG) showed that the oleate-containing DG are less orderly packed than those containing linoleate (32). This too would suggest theoretically that the fluidity of oleate- and linoleate-enriched membranes may be different. However, we observed no change in the cholesterol content or free cholesterol/phospholipid ratio of linoleate-treated cells when compared to oleate controls. These factors most likely account in part for the lack of change in fluidity observed upon linoleate enrichment.

It has been suggested that the activity of 5′-nucleotidase depends on the Sph or PC content of the membrane (33). Interestingly, we observed an increase in the activity of this enzyme despite a reduction in these phospholipids upon DHA enrichment. This would suggest that another mechanism, such as changes in the biophysical properties of the membranes, was responsible for the observed increased activity. For example, an increase in the lipid disorder would allow the enzyme more conformational freedom, and increase its activity by decreasing the energy of activation. It is also possible that a modified lipid environment can lead to stabilization of a more active conformation of the enzyme.

The activity of fluoride-stimulated adenylate cyclase was also increased upon DHA enrichment. The activity of this enzyme is believed to be dependent upon the

membrane cholesterol content (33). Our observations of increased activity of this enzyme and a concomitant decrease in the free cholesterol/phospholipid ratio upon DHA enrichment is at variance with some reported work (34), but in agreement with some other investigations (15). It has been suggested that an optimal lipid environment is necessary for the maximal activity of this enzyme (34), and it is possible that DHA treatment, which decreases both the free cholesterol/phospholipid ratio and membrane viscosity, provides such an environment. The increased disorder in membrane lipids would probably allow a more efficient coupling of adenylate cyclase's regulatory proteins and catalytic subunits. Our observation of no change in the basal activity of the enzyme is in agreement with other studies, as it has been postulated that the basal activity is very resistant to changes in membrane lipid composition (9,15,35).

In conclusion, EPA and DHA have differential effects on membrane lipid composition, fluidity and function of human skin fibroblasts. Recently Bates *et al.* (36) reported that treatment of neutrophils with DHA, but not with EPA, increased the neutrophil-mediated endothelial detachment, showing that these fatty acids exert differential effects on neutrophils also. Our recent work also showed a significant increase in LDL receptor function in DHA- but not in EPA-enriched fibroblasts (E. Brown and P.V. Subbaiah, unpublished observations). Furthermore, recent studies from our laboratory suggested that the positional specificity of plasma lecithin:cholesterol acyltransferase is altered in the presence of 16:0/22:6 PC, but not in the presence of 16:0/20:5 PC, resulting in the synthesis of saturated cholesteryl ester in the presence of the former (37). These differential effects of EPA and DHA on membrane and lipoprotein metabolism may contribute to the known differences in their anti-atherogenic properties (6,7).

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Comparison of Linoleic Acid and Eicosapentaenoic Acid Incorporation into Human Breast Cancer Cells

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To gain some insight into the mechanisms involved in the opposing effects of linoleic acid (LA) and eicosapentaenoic acid (EPA) on the growth and invasiveness of MDA-MB-435 human breast cancer cells, the dynamics of the uptake by cells and the incorporation of [^{14}C]LA and [^{14}C]EPA into major lipid and phospholipid pools, as well as the effects of unlabeled EPA or LA on the uptake and distribution of [^{14}C]LA or [^{14}C]EPA, respectively, were examined. Cells were exposed to [^{14}C]LA (1.28 μg/mL) or [^{14}C]EPA (1.0 μg/mL) and unlabeled EPA or LA, respectively, at 0, 1, 4 and 16 μg/mL for 24 h in serum-free media. The uptake of each fatty acid (FA) was linear over time and was not affected by the presence of the opposing FA. For both FA, 80–90% was incorporated into the phospholipid fraction with the remaining 10–20% in neutral lipids. The relative distribution profile of [^{14}C]LA among the phospholipid classes indicated a preferential incorporation into phosphatidylcholine (65%), whereas [^{14}C]EPA was mostly found in phosphatidylethanolamine (58%). In the presence of unlabeled EPA or LA at various concentrations, corresponding dose-dependent shifts of [^{14}C]LA or [^{14}C]EPA from the phospholipid to the neutral lipid pool were noted, which did not alter the relative distribution of the FA among the phospholipid classes. Exogenous exposure to EPA or LA increased its content in membrane phospholipids while concurrently decreasing LA or EPA content, respectively, in a dose-dependent manner. Arachidonic acid content of membrane phospholipids remained constant. The divergent distribution profiles of LA and EPA within the phospholipid compartment provides some insight into the mechanisms of their opposing effects on MDA-MB-435 cell growth and invasiveness. Also, the effects of LA and EPA on the uptake and distribution of their opposing FA shed some light on the mechanisms mediating their competitive effects.

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Modulation of chemically-induced mammary carcinogenesis by both the level and type of dietary fatty acids (FA) has been demonstrated in rodent models (1–4). Moreover, the growth of transplantable rodent mammary carcinomas has been shown to be stimulated by feeding diets rich in n-6 FA and to be suppressed by n-3 FA-rich diets (5–8). Although several mechanisms may be involved in these dietary effects, most investigators have emphasized a role for prostaglandins and leukotrienes derived from linoleic acid (LA) in stimulating carcinoma development and progression and an inhibitory influence of n-3 FA on eicosanoid biosynthesis (2,4,7).

Studies from our laboratory have indicated (9–15) that FA also influence the growth of human breast cancer cells, as well as expression of the metastatic phenotype. Growth of human breast cancer cell lines in culture was stimulated by LA, an n-6 FA, whereas the n-3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exerted inhibitory effects (9,10); these two classes of FA also had corresponding effects on the capacity of MDA-MB-435 human breast cancer cells to invade through a reconstituted basement membrane in an *in vitro* assay (11). In both situations, these opposing effects of the n-6 and n-3 FA have been associated with inhibition of lipoxygenase-mediated eicosanoid synthesis by the tumor cells (10–12).

Furthermore, a high-fat diet rich in LA stimulated the growth and metastasis of MDA-MB-435 cells when injected into the mammary fat pads of athymic nude mice (13,14), and a high dietary intake of menhaden oil, rich in n-3 FA, suppressed the growth and metastasis of this cell line in the nude mouse model (15).

In addition to direct inhibitory effects on enzymes involved in arachidonate metabolism, the divergent effects of n-6 and n-3 FA on breast cancer growth and metastasis may directly involve the cell membrane phospholipids which play key roles in intracellular signalling, including the generation of eicosanoids (16). Moreover, changes in the FA composition of the cell membrane phospholipids can alter these pathways (17) and, as membrane composition is influenced by dietary FA (18,19), nutritional factors are capable of influencing cell signalling pathways.

The present study was undertaken to define and compare the cellular uptake and the incorporation of LA and EPA into the major phospholipid and neutral lipid pools of MDA-MB-435 cells *in vitro*. The influence of EPA and LA on the uptake and distribution profiles of LA and EPA, respectively, was also established as a preliminary investigation of the competitive effects between n-6 and n-3 FA.

MATERIALS AND METHODS

Materials. [1-^{14}C]Linoleic acid (55 mCi/mmol) and [1-^{14}C]eicosa-5,8,11,14,17-pentaenoic acid (58 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Lipid and phospholipid standards and insulin were purchased from Sigma Chemical Company (St. Louis, MO), and delipidized bovine serum albumin (BSA) from Collaborative Biomedical Products (Bedford, MA). Thin-layer chromatography (TLC) plates were

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BSA, bovine serum albumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid(s); FBS, fetal bovine serum; HETE, hydroxyeicosatetraenoic acid; IMDM, Iscove's modified Dulbecco's medium; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PS, phosphatidylserine; TLC, thin-layer chromatography.

from Whatman Inc. (Clifton, NJ); Triton X-100 (scintillation grade) and Budget-Solve scintillation fluid were obtained from Research Products International Corporation (Mount Prospect, IL). For cell culture, Iscove's modified Dulbecco's medium (IMDM) was obtained from Gibco (Grand Island, NY), fetal bovine serum (FBS) from Upstate Biotechnologies Inc. (Lake Placid, NY) and cell culture plates from Corning (Corning, NY). Solvents were of high purity high-performance liquid chromatography grade.

Cell culture. The MDA-MB-435 human breast cancer cell line, which is estrogen-independent and estrogen receptor negative (20), was provided by J.E. Price (M.D. Anderson Cancer Center, Houston, TX). Cells were cultured in IMDM supplemented with 5% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a 95% air/5% CO_2 incubator. Cell passages from 5 to 28 were used.

Experimental conditions. Cells were plated in 60-mm culture plates at a density of 2.0×10^4 cells/cm^2 and cultured for 24 h in 5% FBS-supplemented IMDM. The medium was then changed to serum-free IMDM containing insulin (10 µg/mL) and delipidized BSA (1.25 mg/mL). For the first experimental set, 1.0 µCi of [^{14}C]LA (final concentration 1.28 µg/mL) was added to each plate. Duplicate plates also received unlabeled EPA at a final concentration of 0.0, 1.0, 4.0 or 16.0 µg/mL. For the second experimental set, a mixture of 0.1 µCi of [^{14}C]eicosa-5,8,11,14,17-pentaenoic acid and unlabeled EPA was added to each plate to yield a final concentration of 1.0 µg/mL. Duplicate plates also received unlabeled LA at a final concentration of 0.0, 1.0, 4.0 or 16.0 µg/mL. Cells were exposed to the FA for 24 h. All FA were prepared in USP ethanol (200 proof), and the final ethanol concentration in the culture media was 0.1%.

For the preparation of cells for FA analysis by gas chromatography, cells were plated in 100-mm culture plates at 2.0×10^4 cells/cm^2 and received unlabeled FA at concentrations stated in the above protocols. Five plates of cells per condition were treated, washed as described later, scraped, and pooled into 500 µL phosphate buffered saline. Aliquots (10 µL) were removed for protein determination (Bio-Rad, Richmond, CA).

Cellular uptake of [^{14}C]fatty acids. Aliquots of media from labeled cells were removed and counted at various time points from 0–24 h to determine FA uptake. Percent uptake was calculated as:

$$\frac{(\text{cpm at time 0}) - (\text{cpm at time point})}{\text{cpm at time 0}} \times 100$$

Results represent the mean ± SE of three experiments performed in duplicate.

Analysis of [^{14}C]fatty acid distribution among cellular phospholipids and neutral lipids. Cells were washed once with serum-free IMDM containing insulin (10 µg/mL) and delipidized BSA to remove unincorporated FA, and twice with PBS. Cells were scraped into 500 µL PBS, and lipids were extracted with 2.25 mL of $CHCl_3$/MeOH (1:2, vol/vol), 0.1 mL distilled H_2O, 0.75 mL $CHCl_3$ and 0.75 mL 4M KCl, according to the method of Bligh and Dyer (21). The $CHCl_3$/MeOH (1:2, vol/vol) solvent mixture contained phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) standards. The extract was dried under N_2, resuspended in 200 µL $CHCl_3$/MeOH (1:2, vol/vol) and applied to a 60A silica gel TLC plate. Phospholipids, neutral lipids and a triglyceride standard were separated using the solvent system chloroform/methanol/acetic acid (65:45:4, by vol) (22). The following R_f values were determined in this solvent system: PC (0.07), PE (0.42), PI/PS (0.22), and triglyceride (neutral lipid; 0.85). Lipids were made visible by exposure to iodine vapors, scraped from the plate, eluted from the silica gel with methanol/Triton X-100 (6:1, vol/vol), and quantitated by scintillation counting. Results represent the mean ± SE of three independent experiments performed in duplicate.

FA analysis by gas chromatography. Lipids were extracted according to the method of Folch *et al.* (23). Briefly, 0.2 volumes of 0.12 M KCl and 4.5 mL of $CHCl_3$/MeOH (2:1, vol/vol) containing 50 µg/mL dipentadecanoyl phosphatidylcholine as internal standard were added to 0.5 mL of cell suspension. The mixture was vortexed for 2 min, centrifuged at $1000 \times g$ for 10 min, and the lower phase containing the lipids was evaporated to dryness under N_2. The residue was reconstituted in 6 mL of acetone saturated with $MgCl_2$ and stored overnight at –20°C. The acetone phase containing neutral lipids was discarded, and the remaining precipitate (phospholipid phase) was redissolved in methanol/benzene (4:1, vol/vol). Transesterification of FA was performed as described by Lepage and Roy (24). The FA methyl esters were separated and quantified by gas chromatography (Hewlett Packard 5890A; Hewlett-Packard, Palo Alto, CA) on a 30 m × 0.25 mm capillary column (Supelco, Bellefonte, PA). Helium was used as carrier gas, and the temperature was programmed from 145 to 181°C at 1°C/min. Individual FA methyl esters were identified by comparison with known standards (Nu-Chek-Prep, Elysian, MN). Single determinations from two independent experiments are presented.

Statistics. Data were analyzed using the SigmaStat program and one-way analysis of variance (ANOVA) or, when necessary, the Kruskal-Wallis ANOVA on Ranks, followed by the Student Newman Keuls Test. Values of $P < 0.05$ were considered significant.

RESULTS

Time-course studies were performed to establish a profile for the uptake of LA and EPA by MDA-MB-435 cells and to determine the effect of competition between the two FA on their respective uptakes. MDA-MB-435 cells steadily accumulated [^{14}C]LA and [^{14}C]EPA during the first 8–16 h of exposure, after which little further accumulation was noted (Fig. 1). The initial rate of cellular uptake was greater for [^{14}C]EPA with 3-fold and 1.6-fold higher rates from 0–4 h and 4–16 h, respectively, as compared to [^{14}C]LA. Uptake was nearly maximal (~90%) at 16 h for both FA; for convenience, the 24-h time point was chosen for subsequent FA incorporation experiments. [^{14}C]LA (1.28 µg/mL) uptake was not af-

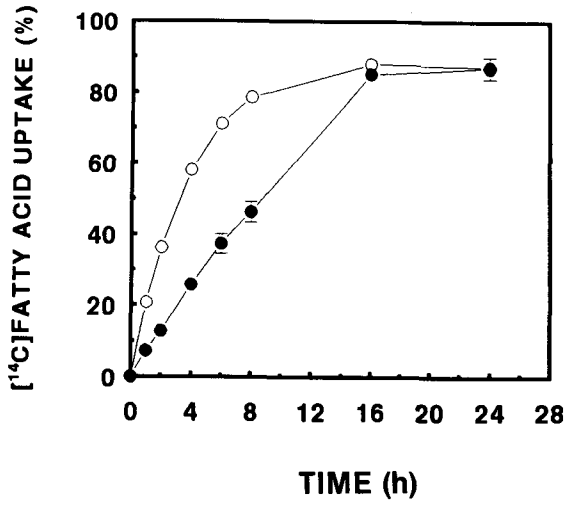

FIG. 1. Time-course of uptake of [^{14}C]linoleic acid (LA) and [^{14}C]eicosapentaenoic acid (EPA) into MDA-MB-435 cells. Cells (2×10^4 cells/cm^2) were treated with 1.0 μCi of [^{14}C]LA (●) or 0.1 μCi of [^{14}C]EPA (○) for 24 h in serum-free media. Aliquots of media were counted as described in the Materials and Methods section to determine percent fatty acid uptakes. Data are expressed as mean ± SE of three experiments performed in duplicate.

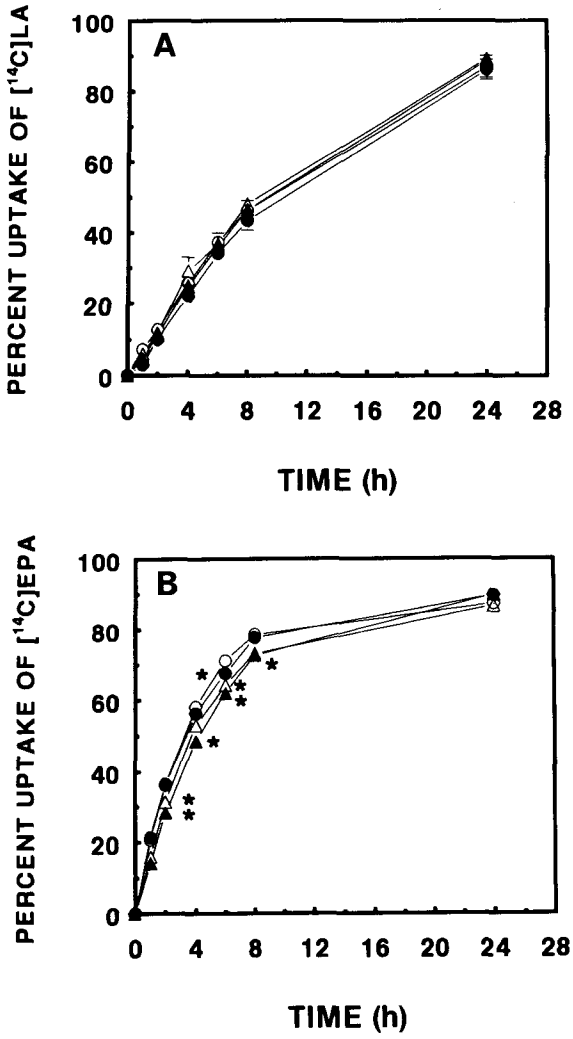

FIG. 2. A. Effect of EPA on the uptake rate of [^{14}C]LA into MDA-MB-435 cells. Cells were exposed to 1.0 μCi of [^{14}C]LA (1.28 μg/mL final concentration) plus 0 (○), 1 (●), 4 (△) or 16 (▲) μg/mL unlabeled EPA for 24 h in serum-free media. Aliquots of media were counted to determine percent [^{14}C]LA uptake. B. Effect of LA on the uptake rate of [^{14}C]EPA into MDA-MB-435 cells. Cells were exposed to 0.1 μCi of [^{14}C]EPA (1.0 μg/mL final concentration) plus 0 (○), 1 (●), 4 (△) or 16 (▲) μg/mL unlabeled LA for 24 h in serum-free media. Aliquots of media were counted to determine percent [^{14}C]EPA uptake. Data represent mean ± SE of three experiments performed in duplicate; *$P < 0.05$ vs. 0 μg/mL LA. Abbreviations as in Figure 1.

fected by exogenous supplementation of EPA at concentrations of 1, 4 or 16 μg/mL (Fig. 2A). Similarly, LA did not substantially reduce the [^{14}C]EPA uptake rate, although a slight dose-dependent inhibition reached statistical significance (Fig. 2B).

Comparison of the degree of esterification of the labeled FA into the two lipid classes revealed similar profiles with 80% of [^{14}C]LA and 93% of [^{14}C]EPA incorporated into phospholipids and 20 and 7% into neutral lipids, respectively (Fig. 3A). However, the relative distribution of incorporated [^{14}C]LA and [^{14}C]EPA differed substantially among the phospholipid fractions. Of the phospholipid-associated [^{14}C]LA, 65% of [^{14}C]LA was incorporated into PC, and 21% and 14% into the PE and PI/PS fractions, respectively. For [^{14}C]EPA, the major phospholipid incorporation site was PE with 58% incorporation, followed by PC with 25% and PI/PS with 17% (Fig. 3B). These proportional distributions for [^{14}C]LA and [^{14}C]EPA within the phospholipid compartment of MDA-MB-435 cells were not altered by concurrent exposure to graded concentrations of unlabeled EPA or LA, respectively (data not shown). Free FA accounted for only 1–3% of the total lipids under all experimental conditions.

The effects of unlabeled EPA and LA on the incorporation of [^{14}C]LA and [^{14}C]EPA, respectively, into the lipid pools of MDA-MB-435 cells were examined next. Exposure of cells to [^{14}C]LA plus graded concentrations of unlabeled EPA (1, 4 and 16 μg/mL) significantly reduced [^{14}C]LA incorporation into the phospholipid fraction in a concentration-dependent manner (Fig. 4A). However, the sensitivity of the effect of EPA on [^{14}C]LA incorporation into phospholipids was not relatively marked since 4-fold and 16-fold greater amounts were required to produce 25 and 60% reductions, respectively. A similar profile was obtained for the effect of unlabeled LA on the incorporation of [^{14}C]EPA into the phospholipid fraction (Fig. 4B), which may suggest that LA and EPA are equally sensitive to the effects of its opposing FA. For both experimental sets, the decreases in phospholipid incorporation were accompanied by corresponding increases of the [^{14}C]FA into the neutral lipid compartment.

Further analyses of the effects of unlabeled EPA and LA on the distribution of [^{14}C]LA and [^{14}C]EPA, respectively, among the three phospholipid fractions (PC, PE, PI/PS) were performed to determine the specific phospholipid involved in attenuation of [^{14}C]FA incorpora-

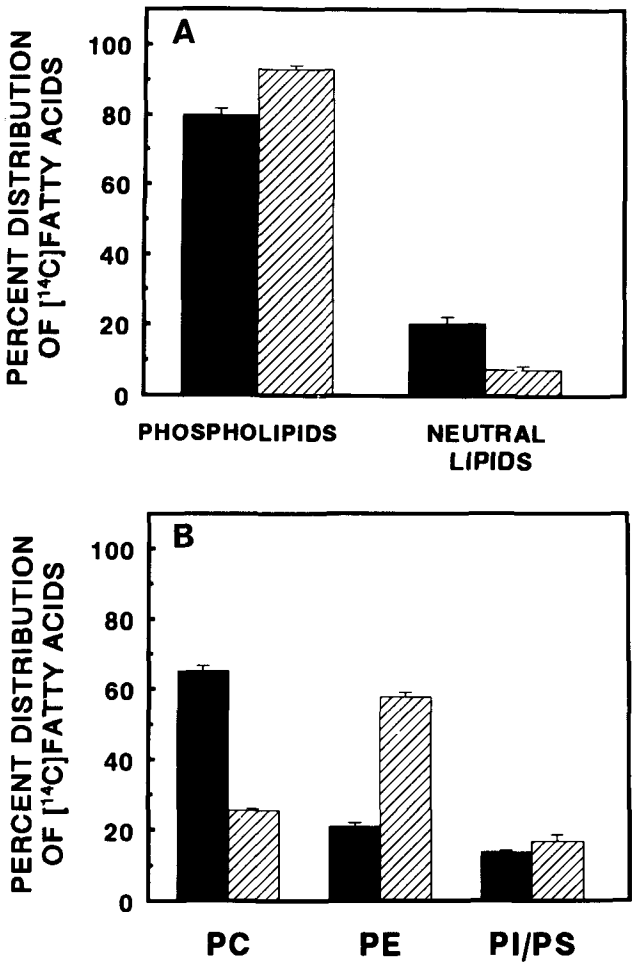

FIG. 3. A. Incorporation of [^{14}C]LA and [^{14}C]EPA into the phospholipid and neutral lipid compartments of MDA-MB-435 cells. Cells were exposed to 1.0 μCi of [^{14}C]LA (solid bar) or 0.1 μCi of [^{14}C]EPA (hatched bar) for 24 h in serum-free media. Lipids were extracted, separated by thin-layer chromatography, and counted to determine the percent incorporation of [^{14}C]LA and [^{14}C]EPA into phospholipids and neutral lipids. B. Percent distribution of [^{14}C]LA (solid bar) and [^{14}C]EPA (hatched bar) among the phospholipid fractions. Data are presented as a percentage of total phospholipids. Values represent mean ± SE of three experiments performed in duplicate. Abbreviations as in Figure 1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI/PS, phosphatidylinositol/phosphatidylserine.

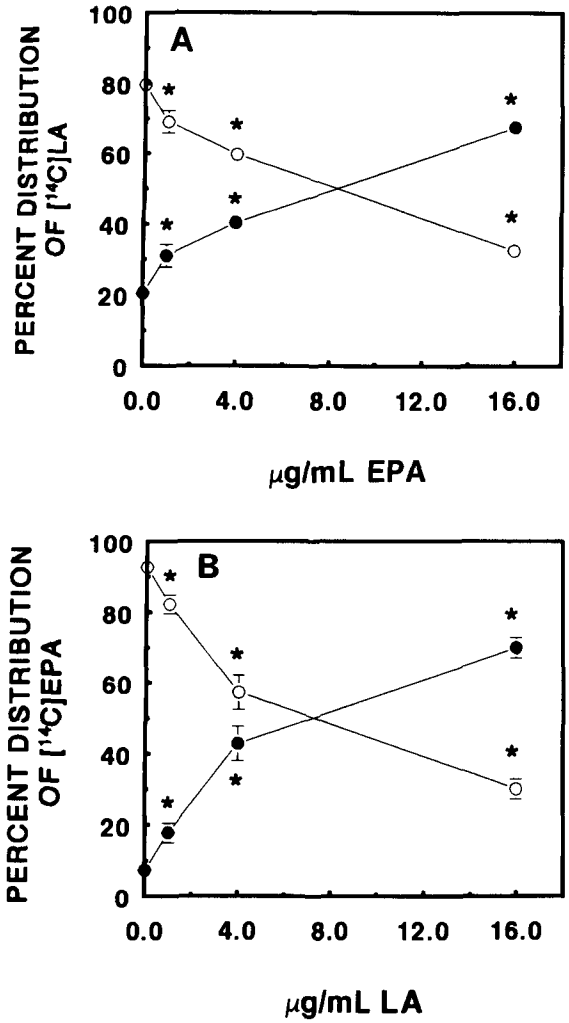

FIG. 4. A. Effect of EPA on [^{14}C]LA incorporation into phospholipid and neutral lipid compartments of MDA-MB-435 cells. Cells were exposed to 1.0 μCi of [^{14}C]LA and unlabeled EPA as described in the Materials and Methods section. Lipids were extracted, separated by TLC, and counted to determine percent incorporation of [^{14}C]LA into phospholipid (○) and neutral lipids (●); *$P < 0.05$ vs. 0 μg/mL EPA. B. Effect of LA on [^{14}C]EPA incorporation into phospholipid and neutral lipid compartments of MDA-MB-435 cells. Cells were exposed to 0.1 μCi of [^{14}C]EPA and unlabeled LA as described in the Materials and Methods section. Lipids were extracted, separated by TLC, and counted to determine percent incorporation of [^{14}C]EPA into phospholipid (○) and neutral lipids (●). Values represent the mean ± SE of three experiments performed in duplicate; *$P < 0.05$ vs. 0 μg/mL LA. Abbreviations as in Figure 1.

tion. Unlabeled EPA dose-dependently decreased [^{14}C]LA incorporation into PC, PE and PI/PS; at the highest dose (16 μg/mL EPA), [^{14}C]LA incorporation was reduced to ~40% (Fig. 5A). Similarly, [^{14}C]EPA incorporation into PC, PE and PI/PS was reduced by unlabeled LA (Fig. 5B) although [^{14}C]EPA appeared more sensitive to the effects of LA as less incorporation (~25%) was observed in the presence of 16 μg/mL LA. For both [^{14}C]LA and [^{14}C]EPA, the decrement of incorporation by its respective opposing FA was relatively equal in each phospholipid fraction.

Table 1 depicts the FA profile of the phospholipid fraction of MDA-MB-435 cells exposed to graded concentrations of LA and EPA. Exposure of cells to 1.3 μg/mL LA, plus increasing concentrations of EPA, resulted in a dose-dependent decrease in the LA content of membrane phospholipids and a corresponding increase in EPA content. Conversely, treatment of cells with 1.0 μg/mL EPA plus graded concentrations of LA produced a dose-related reduction of EPA composition of membrane phospholipids and a corresponding increase in LA content. In both experimental sets, no significant changes in arachidonic acid (AA) content of the phospholipids were noted. Also, the contents of palmitic and stearic acids were not altered. Representation of the data normalized

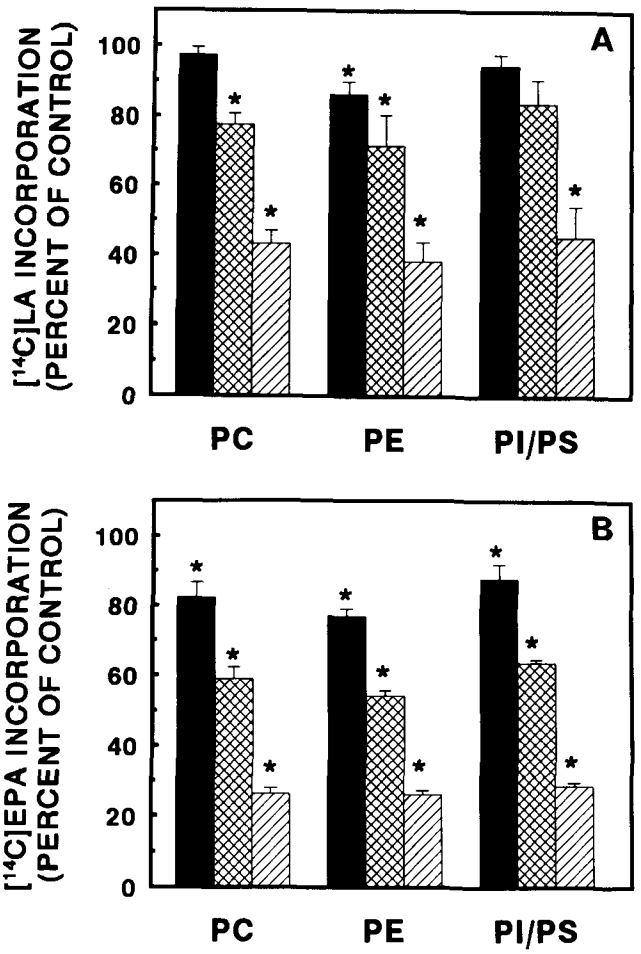

FIG. 5. A. Effect of EPA on the incorporation of [^{14}C]LA into phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol/serine (PI/PS) fractions of MDA-MB-435 cells. Cells were exposed to 1.0 μCi of [^{14}C]LA and 1 (solid bar), 4 (double hatched-bar) or 16 (single-hatched bar) μg/mL unlabeled EPA as described in the Materials and Methods section. The incorporation into each phospholipid fraction is expressed as a percentage of the control (no EPA exposure) set at 100%. B. Effect of LA on [^{14}C]EPA incorporation into PC, PE and PI/PS fractions of MDA-MB-435 cells. Cells were exposed to 0.1 μCi of [^{14}C]EPA and 1 (solid bar), 4 (double-hatched bar), or 16 (single-hatched bar) μg/mL unlabeled LA as described in the Materials and Methods section. The incorporation into each phospholipid fraction is expressed as a percentage of the control (no LA exposure) set at 100%. Values represent the mean ± SE of three experiments performed in duplicate; *$P < 05$ vs. control. Abbreviations as in Figure 1.

to protein yielded the same trends as illustrated in Table 1 (data not shown).

DISCUSSION

As far as we are aware, analyses of the distribution profiles of LA and EPA in cultured human breast cancer cells have not been reported previously. The present study documents the incorporation of LA and EPA into the major lipid pools of MDA-MB-435 cells and demonstrates that this incorporation is altered by the competing FA. LA and EPA were found to be preferentially esterified to phospholipids, which is consistent with the phospholipid pool constituting the major site of FA incorporation in both cultured normal and transformed human cells (25–30).

The primary difference in the distribution profiles of LA and EPA was that LA was preferentially esterified to PC whereas EPA was mostly incorporated into PE. This pattern of distribution may suggest a possible biochemical mechanism for their opposing biological effects, which involve the substrate specificity of phospholipase A_2 (PLA_2), the rate-limiting enzyme in the biosynthesis of eicosanoids. If PC is the preferential substrate for PLA_2 in MDA-MB-435 cells, and PC the primary incorporation site for LA, then PLA_2 activity would result in release of free LA, which would be subsequently converted to AA and ultimately metabolized to series two eicosanoids. Alternatively, if PLA_2 exhibits substrate specificity for PE, the phospholipid which incorporates EPA, then PLA_2-mediated release of EPA would generate series three eicosanoids with their different effects. Therefore, the distinct phospholipid esterification sites of the two FA can potentially lead to the generation of a differing spectrum of eicosanoids with divergent biological actions.

The uptake of the n-6 and n-3 FA was not suppressed by up to 16-fold greater concentrations of the opposing FA, indicating that interference of cellular uptake does not account for the competitive effects of LA and EPA. However, EPA was effective in channeling LA from the phospholipid to the neutral lipid pool; LA similarly altered the distribution of EPA. These results concur with earlier reports of competition between FA for esterification into phospholipids. For example, in cultured fibroblasts, EPA and AA have been shown to independently inhibit incorporation of LA into the total phospholipid fraction while concurrently shunting LA into triacylglycerols (28). Likewise, in neuroblastoma cells, LA incorporation into triacylglycerols increased markedly, and incorporation into phospholipids decreased in the presence of increasing concentrations of 18:3n-3, 18:3n-6, and 20:3n-6 FA (25). It has been suggested that the competition for FA esterification into phospholipids is consistent with a relatively restricted phospholipid acceptor pool which does not expand in response to an influx of exogenous FA (25,28). However, the neutral lipid pool does appear to enlarge to store the excess FA, thereby serving as a cytoplasmic lipid storage site for the displaced FA. In the present study, the shunting of LA or EPA by its opposing FA from the phospholipid compartment, a site involved in intracellular signaling, to a neutral lipid reserve pool suggests an alternate mechanism for the competitive effects of LA and EPA.

A number of investigators have examined the LA and EPA content of chemically-induced mammary carcinomas or transplantable tumor cells in rodents fed diets of differing FA composition; in general, the tumor FA profiles reflected the dietary intakes (2–4,7,31). Jurkowski and Cave (3) reported that tumorigenesis was suppressed as the level of menhaden oil in the diet increased and that levels of EPA and DHA also increased in *N*-nitrosomethylurea-induced rat mammary carcino-

TABLE 1

Content of Linoleic Acid (LA), Eicosapentaenoic Acid (EPA) and Arachidonic Acid (AA) in Phospholipids of MDA-MB-435 Cells Exposed to Graded Concentrations of LA and EPA[a]

Fatty acid treatment (μg/mL)		Fatty acid content (μg/5 plates)					
		LA		EPA		AA	
LA	EPA	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1.3	0.0	24.49	24.18	ND	ND	11.88	10.67
1.3	1.0	21.34	21.77	6.26	5.62	10.49	9.02
1.3	4.0	18.72	21.20	16.80	16.11	8.99	10.19
1.3	16.0	13.75	12.23	31.82	26.38	9.75	6.46
0.0	1.0	3.61	7.50	9.07	7.39	12.78	10.28
1.0	1.0	20.04	19.67	7.58	4.97	10.88	9.58
4.0	1.0	47.37	42.45	2.00	1.88	11.64	8.68
16.0	1.0	82.89	88.07	ND	1.44	7.43	9.29

[a]Cells were plated at 2×10^4 cells/cm^2 (100-mm plates) and treated for 24 h with unlabeled LA and EPA at the indicated concentrations. For each experiment (Expt.), five plates of cells per condition were treated and pooled for fatty acid analysis. Results are reported as μg fatty acid per five plates of cells. Single determinations from two separate experiments are presented. ND, not detected.

mas; there were also corresponding slight decreases in LA and AA. Similarly, Gabor and Abraham (8) demonstrated that feeding fish oil to mice bearing a transplantable mammary adenocarcinoma suppressed tumor growth; also, there was a reduction in the proportion of AA in the tumor total FA content as the n-3 FA increased. In the present study of cultured MDA-MB-435 cells, the relative proportions of LA and EPA in the membrane phospholipids were determined by their concentrations in the medium. Yet, there were no changes in AA, an n-6 FA, which is produced from LA. The relatively short exposure time (24 h) of the cells to the FA may be responsible for the lack of change in AA content. In contrast to this result of a short-term *in vitro* study, we have reported that solid tumors formed by this breast cancer cell line in the mammary fat pads of nude mice show significant reductions in both phospholipid LA and AA, together with prostaglandin E_2, when dietary n-3 FA were increased by feeding a 17% menhaden oil/5% corn oil diet for 12 wk (32).

Growth of estrogen-independent human breast cancer cell lines *in vitro* are stimulated by LA, an effect which is associated with enhanced biosynthesis of lipoxygenase products, principally 12- and 15-(*S*)-hydroxyeicosatetraenoic acids (HETE), and is blocked by esculetin, a selective lipoxygenase inhibitor (10; and Liu, X.-H., Connolly, J.M., and Rose, D.P., manuscript submitted for publication). Moreover, the growth and metastasis of MDA-MB-435 cell solid tumors in athymic nude mice is inhibited by feeding EPA or DHA, and here there are also highly significant reductions in tumor tissue 12-HETE and 15-HETE, together with prostaglandin E_2, concentrations (Rose, D.P., Connolly, J.M., Rayburn, J., and Coleman, M., manuscript submitted for publication). While these observations imply the involvement of the eicosanoids in the opposing effects of dietary n-6 and n-3 FA *in vivo* (14,15), other mechanisms must also be considered. For example, the FA composition of the membrane can alter the activity of membrane-bound enzymes (33), some of which play key roles in intracellular signalling. Also, the cell membrane FA composition determines its fluidity (34) which, in turn, may influence the accessibility of protein hormones (33,35), and perhaps growth factors, to their membrane-associated receptors.

In conclusion, this study establishes the distribution profiles of LA and EPA in a human breast cancer cell line and also defines preliminary competitive effects of the two opposing FA on cellular uptake and phospholipid incorporation. These data provide a first step toward future investigations which will be designed to elucidate the biochemical mechanisms responsible for the divergent effects of LA and EPA on the growth and invasiveness of human breast cancer cells.

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Levels of Polyunsaturated Fatty Acids in Tissues from Zinc-Deficient Rats Fed a Linseed Oil Diet

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The effect of zinc deficiency on the levels of n-6 and n-3 polyunsaturated fatty acids (PUFA) in lipids from tissues of rats fed a diet containing linseed oil was investigated. Rats were fed either a control diet (25 mg Zn/kg) or a zinc-deficient diet (0.8 mg Zn/kg) for 10 d. To avoid energy and nutrient deficiency, 11.6 g of diet per day was administered by gastric tube. At the end of the experiment, rats fed the zinc-deficient diet had drastically reduced plasma zinc concentration and alkaline phosphatase activity consistent with severe zinc deficiency in these rats. Zinc-deficient rats had higher levels of n-3 PUFA, in particular eicosapentaenoic acid (EPA), and lower levels of n-6 PUFA, in particular linoleic acid, in liver and plasma phosphatidylcholine (PC) and in erythrocyte membrane total lipids than did control rats. By contrast, the levels of n-3 PUFA in PC from testes and heart, and in phosphatidylethanolamine (PE) from liver, testes and heart, were only slightly different between zinc-deficient and control rats. The study suggests that desaturation of α-linolenic acid is not inhibited by zinc deficiency, and that in zinc-deficient rats, n-3 PUFA preferentially incorporated into phospholipids at the expense of n-6 PUFA, especially EPA into PC. The study also shows that the effect of zinc deficiency on PUFA levels is different for PC and PE in rat tissues.

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Zinc deficiency has been shown to accentuate essential fatty acid (EFA) deficiency in rats, and an interaction between zinc and EFA metabolism has been proposed (1). A number of studies on the role of zinc in EFA metabolism have been undertaken to examine the effects of zinc deficiency on the fatty acid composition of phospholipids, neutral lipids and total lipids in various tissues (2–10). In some of these studies, zinc deficiency was shown to elevate linoleic acid levels and to lower arachidonic acid levels in rat tissue lipids (3–5,9), and it was thus concluded that zinc plays a role in the desaturation of linoleic acid. This would also be consistent with reports on lowered *in vitro* Δ5 and Δ6 desaturase activities in several tissues of zinc-deficient rats (3,4,11). By contrast, other investigators have reported that arachidonic acid levels were not lowered (6,7), or even elevated (8), in tissues of zinc-deficient rats. The latter studies suggested that zinc would not play a significant role in fatty acid desaturation.

In previous studies interest mainly focused in the effects of zinc on the desaturation of linoleic acid, and thus in most of these experiments rats were fed diets containing oils rich in linoleic acid, such as corn oil (2–6,9) or safflower oil (7). However, fatty acid desaturases do not only catalyze desaturation of fatty acids of the n-6 series, but also catalyze desaturation of n-3 fatty acids. Yet, to our knowledge, the effect of zinc deficiency on the desaturation of fatty acid of the n-3 series has not been investigated. In the present study, rats were fed a zinc-deficient diet containing linseed oil which contains nearly 60% α-linolenic acid, and the levels of n-3 very long chain polyunsaturated fatty acids (PUFA) were examined in various tissue lipids to monitor the effect of zinc deficiency on the desaturation of α-linolenic acid. Thus, the present study should help in clarifying whether zinc deficiency impairs desaturation or not.

It is a common difficulty when studying that rats after a few days on a zinc-deficient diet reduce food intake (12,13). Thus, the effect of zinc deficiency is confounded by undernourishment in experiments in which rats are expected to voluntarily consume zinc-deficient diets (14–17). Reduced food intake is also known to affect desaturation of PUFA (7). In the present study, rats were therefore force-fed by gastric tube to control food intake. Force-feeding has been shown to be a practical approach when studying the effect of short-term zinc deficiency without having results confounded by insufficient nutrient intake (14–18). In a previous study (19) rats were force-fed either a zinc-deficient diet with coconut oil, or a zinc-deficient diet with fish oil. In that study, zinc-deficient rats fed the coconut oil diet developed a fatty liver whereas those fed the fish oil diet did not. This finding suggested that the effect of zinc deficiency on liver lipid levels depends on the dietary fat. Thus, the effect of zinc deficiency on liver lipid levels in rats which were force-fed a diet containing linseed oil was also investigated.

MATERIALS AND METHODS

Animals, diets and tube feeding. Twenty-five male SPF Sprague-Dawley rats weighing 123 ± 6 g (Savo GmbH, Kisslegg, Germany) were divided into two groups. The control group consisted of 12 rats; the depletion group consisted of 15 rats because of the higher risk of mortality. The rats were housed individually in Macrolon cages. A daily 12-h light/dark cycle and a temperature of 23°C and 60% relative humidity were maintained.

All rats were force-fed a semisynthetic diet by gastric tube four times a day (0800, 1300, 1800, 2300) as earlier described in detail (17). The composition of the basic experimental diet is shown in Table 1. The depletion diet contained 0.8 mg Zn/kg, the control diet was supplemented with zinc sulfate to give a zinc concentration of 25 mg/kg. The composition of the linseed oil was as follows (in g/100 g fatty acids): palmitic acid (16:0), 5.3; stearic acid (18:0), 3.7; oleic acid (18:1), 19.7; linoleic acid (18:2n-6), 14.5; and α-linolenic acid (18:3n-3), 56.8; other fatty acids were present only in traces

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Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA polyunsaturated fatty acids; SFA, saturated fatty acids.

TABLE 1

Composition of the Experimental Diet

Ingredient	Amount (g/kg)
Casein	200
Corn starch	328
Sugar	300
Fiber (cellulose)	30
Linseed oil	80
Mineral mixture[a]	40
Vitamin mixture[b]	20
DL-Methionine	2

[a]Minerals per kg diet: $Na_2HPO_4 \cdot 2\ H_2O$, 8.80 g; KH_2PO_4, 8.20 g; KCl, 6.00 g; $MgCl_2 \cdot 6\ H_2O$, 3.40 g; $CaCO_3$, 13.6 g; $FeSO_4 \cdot 7\ H_2O$ 248.8 mg; $CuSO_4 \cdot 7\ H_2O$, 47.2 mg; $MnSO_4 \cdot 5\ H_2O$, 123.1 mg; KI, 9.0 mg; $NiSO_4 \cdot 6\ H_2O$, 4.48 mg; $NaMoO_4 \cdot 2\ H_2O$, 0.50 mg; $SnCl_2 \cdot 2H_2O$, 0.57 mg; $Na_2SeO_3 \cdot 5\ H_2O$, 0.67 mg; $CrCl_3 \cdot 6\ H_2O$, 0.51 mg; NH_4VO_3, 0.23 mg; $Na_2SiO_3 \cdot 5\ H_2O$, 1.51 mg.
[b]Vitamins per kg diet: 1.7 mg all-*trans* retinol; 7.5 µg cholecalciferol; 150 mg all-*rac*-α-tocopherol; 5 mg menadione sodium bisulfite; 5 mg thiamin · HCl; 10 mg riboflavine; 6 mg pyridoxine · HCl; 50 mg Ca pantothenate; 20 mg nicotinic acid; 1000 mg choline chloride; 0.2 mg folic acid; 0.025 mg cyanocobalamin; sugar to 20 g.

(<0.1 g/100 g fatty acids). Diet slurries were freshly prepared before each feeding by mixing the dry dietary components (92 g) with linseed oil (8 g) and 60 mL of doubly distilled water (with or without zinc sulfate). Immediately before feeding, the slurry was heated in a glass bottle at 50°C for a few minutes. The gastric tube consisted of a 5-mL syringe connected to a slide catheter. During tube feeding, the rats were conscious and held by hand. The catheter was inserted into the stomach of the rats, 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 (equal to 11.6 g of food per day). The rats had free access to drinking water (doubly distilled water, supplemented with 0.14 g/L sodium chloride to adjust osmolarity to that of tap water).

After 8 d, the rats fed the depletion diet showed symptoms of zinc deficiency such as sparse and coarse hair, skin lesions around the mouth, eyes and paws, ataxy, and lethargy; the rats appeared severely ill. At days 9 and 10 of the experiment, two rats fed the zinc-deficient diet died. Therefore, the experiment had to be terminated. At day 11 of the experiment 12 h after the last feeding, all rats were killed by decapitation after light diethyl ether anesthesia. Blood was collected from the neck into heparinized tubes, and liver, heart, testes and the mesentery of the small intestine were excised. Plasma and tissue samples were stored at −80°C until analyzed.

Lipid analyses. Lipids of liver, heart, testes, erythrocyte membranes, mesentery of small intestine, and plasma were extracted with *n*-hexane/isopropanol (3:2, vol/vol; containing butylated hydroxytoluene as antioxidant) according to Hara and Radin (20). Prior to extraction, erythrocytes were washed three times with sodium chloride solution (9 g/L), and hemolyzed. Erythrocyte membrane fragments were washed three times with hypotonic Tris buffer, pH 7.4 (21).

Phospholipids of the extract were separated by high-performance liquid chromatography (HPLC) (22) using a Merck-Hitachi (Darmstadt, Germany) system consisting of a gradient pump (L-6200), a diode array (L-3000), a 25 cm x 0.4 cm (internal diameter) Si 60 (5 µm) cartridge (LiChroCART, Merck), an integrator (D-2000), and a fraction collector (Model 201; Gilson, Villiers-le-Bel, France). The gradient system consisted of the mobile phases (i) acetonitrile, (ii) acetonitrile/85% phosphoric acid (99.8:0.2, vol/vol), and (iii) methanol/85% phosphoric acid (99.8:0.2, vol/vol). This method allowed base line separation of all major phospholipid classes. In the present study, phosphatidylcholine (PC) includes all subclasses of choline glycerophospholipids and phosphatidylethanolamine (PE) all ethanolamine glycerophospholipids. All lipids to be analyzed for their fatty acid composition were transmethylated with boron trifluoride/methanol (100 g/L) reagent (23). Fatty acid methyl esters were separated by capillary gas chromatography (Sichromat 2-8; Siemens, Karlsruhe, Germany) using a programmed temperature vaporizing injection system, a flame-ionization detector and a 50 m CP-Sil 88 WCOT capillary column (Chrompack, Middleburg, The Netherlands). Fractions were identified by comparing their retention times with those of individual pure standards, and were quantified relative to heptadecanoic acid methyl ester used as internal standard (24).

For measurement of liver total cholesterol and triglycerides, extracted lipids were dissolved in Triton X-100 as described by De Hoff *et al.* (25). Total cholesterol and triglycerides were determined using enzymatic reagent kits obtained from Boehringer (Mannheim, Germany). Concentrations of liver phospholipids were calculated by the amount and the mean molecular mass of its bound fatty acids after separation of phospholipids by HPLC and gas chromatographic fatty acid analysis (22).

Zinc analyses and activity of alkaline phosphatase. The zinc concentration of blood plasma was measured directly by aspirating a dilute solution (1:5, vol/vol) into a flame of an atomic spectrophotometer (model 5100; Perkin-Elmer, Überlingen, Germany). The activity of alkaline phosphatase (E.C. 3.1.3.1) in plasma was measured using commercial kit reagents (Boehringer) and an auto analyzer (Hitachi, Model 704; Boehringer).

Data analysis. Treatment effects were evaluated by analysis of variance using Minitab (release 7.2) software (MINITAB, Inc., State College, PA).

RESULTS

Growth and zinc status. The initial body weight was 122.5 ± 6.7 g (mean ± SD) for the control rats and 123.2 ± 5.6 g for the rats fed the zinc-deficient diet. During the first eight days of the experiment, the weight gain of the rats fed the two diets was similar; the body weight at day 8 was 154.4 ± 7.2 g for the control rats and 152.1 ± 4.7 g for the zinc-deficient rats. Thereafter, the zinc-deficient rats ceased to grow, whereas the control rats continued their weight gain. The final body weight at day 11 was 169.5 ± 6.6 g for the control rats and 152.0 ± 6.6 g for the zinc-deficient rats ($P < 0.05$). The zinc concentration in plasma was 15.3 ± 2.0 µmol/L in the control rats and 4.4 ± 0.6 µmol/L in the zinc-deficient rats. The

TABLE 2

Fatty Acid Composition of Phosphatidylcholine from Liver and Plasma of Control and Zinc-Deficient Rats[a]

Fatty Acid	Liver		Plasma	
	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)
Σ Saturated	44.44 ± 1.06	45.46 ± 1.93	45.58 ± 0.98[b]	47.97 ± 2.14[c]
Σ Monounsaturated	10.84 ± 0.74[b]	9.67 ± 0.90[c]	10.24 ± 0.78[b]	8.86 ± 0.80[c]
18:2n-6	14.06 ± 1.13[b]	10.30 ± 1.98[c]	19.12 ± 1.13[b]	14.93 ± 2.54[c]
Metabolites				
18:3n-6	0.16 ± 0.03	0.18 ± 0.03	0.10 ± 0.03	0.13 ± 0.04
20:3n-6	0.91 ± 0.10[b]	0.62 ± 0.14[c]	1.05 ± 0.14[b]	0.66 ± 0.11[c]
20:4n-6	10.86 ± 0.53	10.40 ± 0.92	10.10 ± 0.60	9.69 ± 1.13
22:4n-6	0.09 ± 0.04	0.09 ± 0.03	0.10 ± 0.03	0.08 ± 0.02
22:5n-6	0.15 ± 0.05	0.13 ± 0.03	0.21 ± 0.08	0.20 ± 0.06
18:3n-3	1.81 ± 0.25[b]	2.61 ± 0.36[c]	1.64 ± 0.30[b]	2.66 ± 0.75[c]
Metabolites				
20:5n-3	7.46 ± 0.74[b]	11.85 ± 1.21[c]	4.91 ± 0.54[b]	9.69 ± 1.13[c]
22:5n-3	2.19 ± 0.46[b]	1.82 ± 0.23[c]	2.07 ± 0.42[b]	1.64 ± 0.25[c]
22:6n-3	5.87 ± 0.73	5.90 ± 0.86	4.31 ± 0.46	4.09 ± 0.82
Σ (n-6)	26.52 ± 1.06[b]	21.91 ± 2.66[c]	31.02 ± 1.04[b]	25.92 ± 1.97[c]
Σ (n-3)	17.84 ± 1.46[b]	22.70 ± 1.38[c]	13.16 ± 0.91[b]	17.21 ± 1.47[c]
Σ (n-6)/Σ(n-3)	1.50 ± 0.16[b]	0.97 ± 0.17[c]	2.37 ± 0.19[b]	1.52 ± 0.20[c]

[a]Mean ± SD (n = 12 for control group; n = 13 for zinc-deficient group). Means were compared by pairs (Zn^+ vs. Zn^-) within one tissue. Values with different superscripts (b, c) are significantly different ($P < 0.05$).

activity of alkaline phosphatase in plasma was 397 ± 71 Units/L in the control rats and 163 ± 48 Units/L in the zinc-deficient rats.

Fatty acid composition of lipids. The fatty acid composition of PC from liver and plasma is shown in Table 2. The effect of zinc deficiency on fatty acid composition was similar in liver and plasma PC. Total monounsaturated fatty acids (MUFA), linoleic acid (18:2n-6), dihomo-γ-linolenic acid (20:3n-6), docosapentaenoic acid (DPA, 22:5n-3) and total n-6 PUFA were lower in liver and plasma PC of zinc-deficient rats; α-linolenic acid (18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and total n-3 PUFA were elevated in liver and plasma PC of zinc-deficient rats. In plasma PC, total saturated fatty acids

TABLE 3

Fatty Acid Composition of Phosphatidylcholine from Testes and Heart of Control and Zinc-Deficient Rats[a]

Fatty acid	Testes		Heart	
	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)
Σ Saturated	47.70 ± 0.39	47.88 ± 1.40	41.16 ± 0.93	41.02 ± 0.85
Σ Monounsaturated	18.32 ± 0.40[b]	19.01 ± 0.48[c]	10.14 ± 0.61	10.07 ± 0.79
18:2n-6	4.79 ± 0.27	4.60 ± 0.45	11.84 ± 1.56[b]	13.61 ± 1.74[c]
Metabolites				
18:3n-6	0.18 ± 0.07	0.16 ± 0.06	0.05 ± 0.02	0.05 ± 0.01
20:3n-6	1.31 ± 0.11	1.34 ± 0.12	0.49 ± 0.07	0.45 ± 0.04
20:4n-6	12.23 ± 0.33[b]	11.80 ± 0.52[c]	20.56 ± 0.83[b]	18.30 ± 0.91[c]
22:4n-6	0.85 ± 0.05	0.82 ± 0.06	0.43 ± 0.04	0.44 ± 0.03
22:5n-6	10.86 ± 0.40	10.47 ± 0.69	0.31 ± 0.07	0.35 ± 0.10
18:3n-3	0.14 ± 0.05	0.15 ± 0.04	1.36 ± 0.18[b]	1.64 ± 0.22[c]
Metabolites				
20:5n-3	0.41 ± 0.05[b]	0.56 ± 0.10[c]	1.81 ± 0.20	1.93 ± 0.20
22:5n-3	0.14 ± 0.01[b]	0.16 ± 0.02[c]	4.22 ± 0.48	4.23 ± 0.55
22:6n-3	1.86 ± 0.15	1.82 ± 0.26	6.61 ± 0.56	6.97 ± 0.97
Σ (n-6)	30.59 ± 0.43[b]	29.60 ± 1.18[c]	33.95 ± 1.15	33.43 ± 1.30
Σ (n-3)	2.60 ± 0.18	2.74 ± 0.29	14.22 ± 0.74	15.00 ± 1.24
Σ (n-6)/Σ (n-3)	11.84 ± 0.88[b]	10.89 ± 1.06[c]	2.40 ± 0.16	2.25 ± 0.27

[a]Mean ± SD (n = 12 for control group; n = 13 for zinc-deficient group). Means were compared by pairs (Zn^+ vs. Zn^-) within one tissue. Values with different superscripts (b,c) are significantly different ($P < 0.05$).

TABLE 4

Fatty Acid Composition of Phosphatidylethanolamine from Liver, Testes and Heart of Control and Zinc-Deficient Rats[a]

	Liver		Testes		Heart	
Fatty acid	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)
Σ Saturated	45.27 ± 0.78[b]	46.35 ± 1.16[c]	32.34 ± 0.60	32.08 ± 0.76	42.70 ± 1.07	42.98 ± 0.82
Σ Monounsaturated	4.93 ± 0.54	5.19 ± 0.74	10.43 ± 0.82[b]	11.23 ± 0.78[c]	6.28 ± 0.59	6.27 ± 0.72
18:2n-6	5.75 ± 0.62[b]	4.52 ± 0.99[c]	3.44 ± 0.23[b]	2.97 ± 0.28[c]	6.39 ± 0.79	7.04 ± 0.84
Metabolites						
18:3n-6	0.06 ± 0.01[b]	0.07 ± 0.01[c]	—	—	—	—
20:3n-6	0.45 ± 0.05[b]	0.33 ± 0.09[c]	0.77 ± 0.07[b]	0.70 ± 0.08[c]	0.26 ± 0.03	0.26 ± 0.04
20:4n-6	11.41 ± 0.77[b]	11.55 ± 0.73[c]	21.63 ± 0.67	21.38 ± 1.12	15.84 ± 0.91	15.48 ± 1.23
22:4n-6	0.09 ± 0.02	0.09 ± 0.01	2.58 ± 0.17[b]	2.45 ± 0.06[c]	0.53 ± 0.17	0.56 ± 0.07
22:5n-6	0.14 ± 0.07	0.18 ± 0.05	21.68 ± 0.63	21.57 ± 0.82	1.07 ± 0.12	1.07 ± 0.10
18:3n-3	1.18 ± 0.20[b]	2.63 ± 0.50[c]	0.15 ± 0.03	0.15 ± 0.02	1.30 ± 0.18[b]	1.47 ± 0.21[c]
Metabolites						
20:5n-3	10.97 ± 0.68	11.53 ± 1.23	0.70 ± 0.08[b]	0.85 ± 0.10[c]	1.24 ± 0.17	1.23 ± 0.17
22:5n-3	4.29 ± 0.75[b]	3.49 ± 0.48[c]	0.44 ± 0.06	0.46 ± 0.10	4.48 ± 0.51	4.61 ± 0.52
22:6n-3	14.54 ± 1.30[b]	13.27 ± 1.51[c]	4.40 ± 0.40	4.75 ± 0.47	18.91 ± 1.67	18.15 ± 2.17
Σ (n-6)	18.08 ± 1.15[b]	16.85 ± 1.26[c]	50.37 ± 1.20[b]	49.35 ± 1.11[c]	24.30 ± 1.30	24.57 ± 2.01
Σ (n-3)	31.34 ± 1.42	31.30 ± 1.36	5.69 ± 0.43[b]	6.21 ± 0.50[c]	26.04 ± 1.45	25.56 ± 1.95
Σ (n-6)/Σ (n-3)	0.58 ± 0.06	0.54 ± 0.05	8.90 ± 0.71[b]	8.00 ± 0.70[c]	0.94 ± 0.09	0.97 ± 0.15

[a]Mean ± SD (n = 12 for control group; n = 13 for zinc-deficient group). Means were compared by pairs (Zn^+ vs. Zn^-) within one tissue. Values with different superscripts (b, c) are significantly different ($P < 0.05$).

(SFA) were also higher in zinc-deficient rats than in control rats, and zinc-deficient rats had a decreased total n-6/n-3 PUFA ratio in liver and plasma PC.

The fatty acid composition of PC from testes and heart is shown in Table 3. The effects of zinc deficiency on the fatty acid composition of testes and heart PC were less pronounced than the effects on the fatty acid composition of liver and plasma PC. Total MUFA, EPA (20:5n-3) and DPA (22:5n-3) were significantly elevated in testes PC of zinc-deficient rats, whereas arachidonic acid (20:4n-6) and total n-6 PUFA were lower. The total n-6/n-3 PUFA ratio in testes PC was lower in zinc-deficient rats than in control rats. The only effect of zinc deficiency on heart PC seen was an elevation of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3).

The fatty acid composition of PE from liver, testes and

TABLE 5

Fatty Acid Composition of Total Lipids from Erythrocyte Membranes and Mesentery of Small Intestine of Control and Zinc-Deficient Rats[a]

	Erythrocyte membranes		Mesentery of small intestine	
Fatty acid	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)	Control (mol/100 mol)	Zinc-deficient (mol/100 mol)
Σ Saturated	48.79 ± 0.97	48.82 ± 1.32	34.12 ± 1.74	34.50 ± 0.91
Σ Monounsaturated	13.79 ± 1.41	13.98 ± 1.07	34.94 ± 1.15	35.78 ± 1.62
18:2n-6	8.81 ± 0.72[b]	7.77 ± 0.83[c]	13.48 ± 0.77	12.96 ± 1.06
Metabolites				
18:3n-6	0.07 ± 0.02[b]	0.09 ± 0.02[c]	0.06 ± 0.01	0.08 ± 0.03
20:3n-6	0.46 ± 0.04	0.40 ± 0.10	0.10 ± 0.03	0.10 ± 0.02
20:4n-6	14.64 ± 1.07	14.66 ± 1.11	0.24 ± 0.09	0.24 ± 0.04
22:4n-6	0.80 ± 0.09	0.83 ± 0.08	—	—
22:5n-6	0.52 ± 0.04	0.53 ± 0.05	—	—
18:3n-3	1.66 ± 0.14[b]	1.92 ± 0.20[c]	16.00 ± 1.19	15.14 ± 1.48
Metabolites				
20:5n-3	2.84 ± 0.34[b]	3.24 ± 0.27[c]	0.20 ± 0.07	0.24 ± 0.04
22:5n-3	3.28 ± 0.28	3.21 ± 0.35	0.27 ± 0.07	0.30 ± 0.03
22:6n-3	3.03 ± 0.26	3.04 ± 0.29	0.16 ± 0.07	0.19 ± 0.04
Σ (n-6)	25.62 ± 1.23	24.63 ± 1.88	14.10 ± 0.83	13.62 ± 1.09
Σ (n-3)	11.03 ± 0.67	11.64 ± 0.72	16.84 ± 1.35	16.10 ± 1.49
Σ (n-6)/Σ (n-3)	2.33 ± 0.11[b]	2.12 ± 0.18[c]	0.84 ± 0.10	0.86 ± 0.13

[a]Mean ± SD (n = 12 for control group; n = 13 for zinc-deficient group). Means were compared by pairs (Zn^+ vs. Zn^-) within one tissue. Values with different superscripts (b, c) are significantly different ($P < 0.05$).

TABLE 6

Concentrations of Lipids in Liver of Control and Zinc-Deficient Rats[a]

Lipid	Control (mg/g liver)	Zinc-deficient (mg/g liver)
Triglycerides	29.1 ± 9.5	28.1 ± 13.4
Total cholesterol	2.49 ± 0.53[b]	2.01 ± 0.42[c]
Phosphatidylcholine	12.1 ± 1.2[b]	13.6 ± 1.1[c]
Phosphatidylethanolamine	7.37 ± 0.79	7.06 ± 1.02

[a]Mean ± SD (n = 12 for control group; n = 13 for zinc-deficient group). Values with different superscripts (b, c) are significantly different ($P < 0.05$).

heart is shown in Table 4. In general, the effects of zinc deficiency on PE were less pronounced than those on PC. Total SFA, γ-linolenic acid (18:3n-6) and α-linolenic acid (18:3n-3) were elevated in liver PE of zinc-deficient rats, whereas linoleic (18:2n-6), dihomo-γ-linolenic acid (20:3n-6), docosatetraenoic acid (22:4n-6) and total n-6 PUFA were lower. Testes PE from zinc-deficient rats contained higher levels of total MUFA, EPA (20:5n-3) and total n-3 PUFA and lower levels of linoleic acid (18:2n-6), dihomo-γ-linolenic acid (20:3n-6), docosatetraenoic acid (22:4n-6) and total n-6 PUFA than that from control rats. Zinc-deficient rats also had a lower total n-6/n-3 PUFA ratio for testes PE. In heart PE, the only change seen due to zinc deficiency was a slight elevation of α-linolenic acid.

The fatty acid composition of total lipids from erythrocyte membranes and the mesentery of small intestine is shown in Table 5. In erythrocyte membrane total lipids of zinc-deficient rats, linoleic acid (18:2n-6) was lower, but total n-3 PUFA and, in particular, α-linolenic acid (18:3n-3) and EPA (20:5n-3) were elevated. Zinc-deficient rats also had a lower total n-6/n-3 PUFA ratio for erythrocyte membrane lipids. In contrast, zinc deficiency did not change the fatty acid composition of total lipids from mesentery of small intestine.

Liver lipids levels. Control rats and zinc-deficient rats had similar liver triglyceride and PE concentrations (Table 6). By contrast, total cholesterol was lower in zinc-deficient rats than in control rats, and PC was higher in zinc-deficient rats than in control rats.

DISCUSSION

The present study was undertaken to investigate the effect of zinc deficiency on the fatty acid patterns of rats fed a diet rich in α-linolenic acid. For this purpose, a rat model was used that permits induction of a severe zinc deficiency without having the observed effects due to zinc deficiency distorted by changes due to energy and nutrient intake. The disadvantage of the model is that it can only be used for short-term studies because rats force-fed a diet containing extremely low zinc concentrations cannot survive beyond 10 or 12 d. In zinc-deficient rats that consumed the diet *ad libitum,* plasma alkaline phosphatase activity, which is an approximate indicator of the zinc state (26,27), was greatly reduced already after 2 to 4 d on a zinc-deficient diet (28,29). This means that a zinc-deficient state existed only for approximately 6 to 8 d. Although the model we used may be physiologically less relevant, it is useful to study the effects of very severe zinc deficiency. In physiologically more relevant models, the effects of zinc deficiency on fatty acid metabolism are typically being investigated using moderately zinc-deficient rats (9), which typically consume adequate quantities of food. However, effects due to severe zinc deficiency might remain hidden when using these models.

In the present study the effect of zinc deficiency on fatty acid metabolism was examined by following its effect on the fatty acid composition of PC and PE. These two phospholipid classes represent approximately 80% of the total phospholipids in rat liver, heart and testes and approximately 90% of total phospholipids in rat plasma (30), and most of the PUFA is associated with there phospholipid fractions (31). In general, fatty acid compositions can be modified by a variety of processes (31), and changes in fatty acid compositions alone do not allow specific conclusions to be drawn about any of the individual processes. Nevertheless, the observation that the levels of EPA in liver phospholipids were unchanged or even elevated suggests that zinc deficiency does not affect Δ5 and Δ6 desaturation of α-linolenic acid, at least not within the time frame of the present study. This disagrees with the results of some earlier studies which showed that zinc deficiency inhibited Δ5 and Δ6 desaturation (3–5,9,11) in rats fed diets containing oils rich in n-6 fatty acids. In the present study, the effects of zinc deficiency on the fatty acid composition of phospholipids were more pronounced in liver and plasma than in other tissues investigated. This might be due to the higher turnover rates of phospholipids in liver than in other tissues (32).

An interesting observation in the present study was that zinc-deficient rats had elevated levels of EPA in PC but not in PE. This could indicate that zinc deficiency may alter the incorporation of EPA into PC. Increased levels of EPA in PC were recently also observed in zinc-deficient rats which had been force-fed a fish oil diet (19). By contrast, the levels of α-linolenic acid were higher in both PC and PE. However, in this and recent studies (18,19), independent of the dietary fat, the levels of linoleic acid were reduced in rats which had been force-fed with zinc-deficient diets. This is in contrast to results obtained on rats that consumed a zinc-deficient diet *ad libitum* (4,5,9,33). The lower levels of linoleic acid we observed in the present study may be due to increased oxidation. The increased levels of linoleic acid which have been reported in the literature were suggested to be due to reduced desaturation (4,5,9,33).

The level of arachidonic acid, which is often used as a measure of desaturation (5,9,33), was not changed by zinc deficiency in either PC or PE. However, it should be noted that feeding a diet rich in α-linolenic acid is known to suppress the desaturation of linoleic acid (34) which is reflected by lower levels of arachidonic acid compared with rats fed diets containing fats with lower levels of n-3 fatty acids (3–9,33).

In a previous study, rats which were force-fed a zinc-deficient diet based predominately on coconut oil developed fatty livers, whereas rats fed a zinc-deficient diet based predominately on fish oil did not (19). In the present study, total lipid and triglyceride levels in liver were not different between control and zinc-deficient rats. This suggests that linseed oil, like fish oil, can prevent the formation of fatty liver which has been observed in rats force-fed zinc-deficient diets based on coconut oil.

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The n-3 Polyunsaturated Fatty Acid Levels in Rat Tissue Lipids Increase in Response to Dietary Olive Oil Relative to Sunflower Oil

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In the present study, changes in phospholipid compositions of liver microsomes, erythrocyte membranes, platelets, aorta, cardiac muscle and brain of rats fed olive oil were compared with those of rats fed sunflower oil. Four groups of rats starting at weaning were fed for four weeks a basal diet containing 5 or 25% olive oil or sunflower oil. We found that oleic acid was higher and linoleic acid was lower in membrane phospholipids of olive oil fed rats compared to sunflower oil fed rats. Polyunsaturated fatty acids of the n-3 series were markedly elevated in all tissues of rats on the olive oil diets relative to those on the sunflower oil diets. The results are consistent with a lower linoleic/linolenic acid ratio induced by the olive oil diets, suggesting a positive correlation between olive oil ingestion and n-3 polyunsaturated fatty acid levels in cell and tissue lipids. The study suggests that an adequate intake of olive oil may enhance the conversion of n-3 fatty acids.

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Long-chain polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series are of much current interest. The intake of PUFA of both series has been correlated with a decreased risk of cardiovascular disease due to the hypolipemic, antithrombotic and antiarrhythmic effects of PUFA (1–3). Furthermore, specific roles for n-3 PUFA have been recognized in specialized tissues, such as brain and retina (4–6).

Changes in cell membrane lipid composition induced by dietary supplementation with unsaturated fats have been repeatedly described (7–13). Nevertheless, there is a growing concern that habitual intake of large quantities of PUFA may be unhealthy, as PUFA are quite susceptible to peroxidation, and thus may promote carcinogenesis and possibly reduce high density lipoprotein levels (14,15).

Recently, attention has been paid to the intake of olive oil with its high oleic acid (18:1n-9) and moderate linoleic acid (18:2n-6) levels. Olive oil is an important component of the so-called Mediterranean diet. Numerous studies have noted that the incidence of cardiovascular disease in the Mediterranean area is one of the lowest of the Western hemisphere (16,17), which has focused renewed interest on olive oil. Decreased plasma cholesterol levels, lower levels of low density lipoprotein (LDL)-cholesterol and thromboxane A_2 (TXA_2), and increased plasma levels of prostacyclin have been reported by us and others after olive oil ingestion (18–22).

A recent study by our group, in which we observed an increase in long-chain n-3 PUFA in liver microsomal fractions from rats fed an olive oil diet rather than a corn oil diet (8) has prompted us to further focus on this olive oil effect. Results of others have also shown increases in LDL n-3 PUFA in individuals on a monounsaturated diet as compared to individuals on a PUFA diet (9). Thus, the aim of the present study was to evaluate the effect of feeding olive oil to rats on the phospholipid (PL) fatty acid compositions of liver microsomes, erythrocyte membranes, platelets, aorta, cardiac muscle and brain. Sunflower oil added to the diet at the same percentages served as reference oil.

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Abbreviations: LDL, low density lipoprotein; O-5, 5% olive oil diet; O-25, 25% olive oil diet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated fatty acids; S-5, 5% sunflower oil diet; S-25, 25% sunflower oil diet; TXA_2, thromboxane A_2.

MATERIALS AND METHODS

Animals and diets. Male weanling Wistar rats (Interfauna, Barcelona, Spain) weighing 50–55 g were housed in wire-bottom cages using a 12-h light/dark cycle. Animals were housed and handled in compliance with our Institution's guidelines for animal research.

Rats were randomly assigned to four groups, each containing ten animals. Two groups were fed similar low-fat diets supplemented with 5% sunflower oil (S-5) or 5% olive oil (O-5). The other two groups were fed high-fat diets that were isocaloric with the low-fat diets, but were supplemented with 25% sunflower oil (S-25) or 25% olive oil (O-25) (Table 1) (22,23). The fatty acid composition of the diets is shown in Table 2. Diets were stored at 4°C, and the rats were fed fresh food daily. Food and water were provided *ad libitum*.

Sample collection. Rats were killed by decapitation under light diethyl ether anesthesia. Blood was collected separately for the isolation of erythrocyte membranes and for the preparation of platelet suspensions. For the former, blood was collected in heparinized tubes, and for the latter

TABLE 1

Composition of the Basal Diets[a]

Component	Low-fat diets	High-fat diets
	(g/100 g diet)	
Cellulose	5.0	30.0
DL-Methionine	0.3	0.3
Casein	20.0	20.0
Starch	49.09	14.63
Sucrose	15.0	4.46
Vitamin mix	1.0	1.0
Mineral mix	4.5	4.5
Choline chloride	0.11	0.11
Olive oil[b]	5.0 (diet O-5)	25.0 (diet O-25)
Sunflower oil[c]	5.0 (diet S-5)	25.0 (diet S-25)

[a]Abbreviations: O-5, olive oil 5%; S-5, sunflower oil 5%; O-25, olive oil 25%; S-25, sunflower oil 25%. The composition of the diets was adjusted so that all animals in all dietary groups would consume the same amounts of protein, minerals, vitamins and calories. An inert ingredient (cellulose) was added to high-fat diets to facilitate diet elaboration.
[b]Carbonell; Carbonell y C.I.A. de Cordoba, S.A., Cordoba, Spain.
[c]Koipesol; Koipe S.A., San Sebastian, Spain.

TABLE 2

Fatty Acid Composition of the Diets[a]

Fatty acid	Olive oil diets	Sunflower oil diets
16:0	12.0	7.3
18:0	3.0	3.7
20:0	0.4	0.2
16:1n-7	1.0	0.2
18:1n-9	74.3	21.0
18:2n-6	8.3	66.7
20:3n-6	—	0.6
18:3n-3	1.0	0.3
18:2n-6/18:3n-3	8.3	222.3

[a]Values are percent distributions of fatty acid methyl esters.

it was collected in 3.8% sodium citrate (1:9, vol/vol) as described by Hashimoto *et al.* (24).

Aorta, brain and heart ventricles were washed in 0.15M NaCl, frozen in liquid nitrogen, and stored at –80°C until analyzed. Liver was washed in 0.15M NaCl and homogenized in phosphate buffer to isolate microsomes.

Analytical methods. To obtain erythrocyte membranes, blood samples were centrifuged at 3000 × *g* for 10 min to separate plasma from cells. Erythrocyte membranes were obtained according to the method described by Hanahan and Ekholm (25). Liver microsomes were isolated according to Philipp and Shapiro (26).

Total lipids were extracted (27) with chloroform/methanol (2:1, vol/vol) containing the antioxidant butylated hydroxytoluene (50 mg/L).

PL were separated by thin-layer chromatography on Silica Gel G 60 (Merck, Darmstadt, Germany) using two different solvent systems, namely hexane/diethyl ether/acetic acid (80:20:1, by vol) (28) to isolate total PL and ethyl acetate/chloroform/*n*-propanol/methanol/0.25% KCl (25:25:25:10:9, by vol) (29) to isolate the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions from liver microsomes.

Fractions were detected by exposure to iodine vapors. The areas of silica gel containing PL, PC and PE were immediately (after iodine evaporation) scraped from the plates, eluted with chloroform, and analyzed for fatty acid composition. Fatty acids were transmethylated according to Morrison and Smith (30).

Fatty acid methyl esters were separated on a 30 m × 0.15 μm (i.d.) fused silica capillary column (DB23 30N; J&W Scientific, Folsom, CA) attached to a Konik 3000 HRGC chromatograph (Barcelona, Spain) equipped with a Spectraphysics SP4290 integrator (San Jose, CA). Fatty acids were identified by comparing retention times with those of standards (Sigma Química, Barcelona, Spain). Fatty acid compositions are expressed as the percent distribution of fatty acid methyl esters.

Statistical analysis. All results are shown as means ± SEM. Analysis with Bonferroni's test for multiple pairwise comparisons was performed. Analyses were done using the BMDP Computer Software Program (BMDP Statistical Software, Cork, Ireland).

RESULTS

The relative fatty acid compositions of the PL of erythrocyte membranes, platelets, cardiac muscle, brain, aorta, liver microsomes and of the two major PL fractions, PE and PC, of liver microsomes are shown in Tables 3 to 6.

The saturated fatty acid compositions are shown in Table 3. Rats fed the sunflower oil diets showed, in general, the highest levels of total saturated fatty acids especially in the high-fat fed group.

The respective figures for oleic and linoleic acids are shown in Table 4. The percentages of oleic acid were markedly greater in animals fed olive oil diets than those of the other two groups, except for brain where there was no effect of diet. Rats fed high-fat diets showed higher oleic acid levels in platelet PL when compared to rats fed low-fat diets; conversely, oleic acid was significantly lower in PL, PC and PE of liver microsomes and cardiac muscle from high-fat vs. low-fat fed rats. The two groups of rats fed sunflower oil diets showed increases in linoleic acid levels which were significant relative to those in the olive oil groups in all the cell sources. Linoleic acid was very low

TABLE 3

Total Saturated Fatty Acids of Phospholipids from Different Sources in Olive Oil or Sunflower Oil Fed Rats[a]

	Diets[b]			
Sources	O-5	O-25	S-5	S-25
Erythrocyte PL	42.5 ± 0.5[d]	42.6 ± 0.6	47.2 ± 0.4	46.7 ± 0.5[c]
Platelet PL	47.5 ± 0.7	47.9 ± 1.2	47.5 ± 1.0	50.1 ± 1.1
Liver microsome PL	42.4 ± 0.4	44.3 ± 0.7	43.0 ± 0.4	47.0 ± 0.6[c,d]
Liver microsome PE	41.3 ± 0.7	45.0 ± 0.7	45.3 ± 1.5	45.4 ± 1.2
Liver microsome PC	44.4 ± 1.3	45.6 ± 0.4	45.2 ± 1.1	47.7 ± 0.5
Heart PL	38.6 ± 0.3[c,d]	40.6 ± 0.2	40.8 ± 0.1	42.4 ± 0.2[c,d]
Brain PL	48.2 ± 0.5	49.1 ± 0.7	52.2 ± 2.5	53.9 ± 1.8
Aortic PL	43.1 ± 1.3	39.1 ± 1.8	41.7 ± 0.9	40.6 ± 1.5

[a]Results are mean percentage distributions ± SEM of fatty acid methyl esters measured on ten animals. PL, phospholipid(s); PE, phosphatidylethanolamine; PC, phosphatidylcholine. [b]See Table 1 for diet abbreviations. [c]$P < 0.05$, significance relative to O-25 group. [d]$P < 0.05$ significance relative to S-5 group.

TABLE 4

Oleic Acid and Linoleic Acid in Phospholipids from Different Sources in Olive Oil or Sunflower Oil Fed Rats[a]

Sources	Fatty acids	Diets[b]			
		O-5	O-25	S-5	S-25
Erythrocyte PL	18:1	19.0 ± 0.6[d]	20.4 ± 0.2	12.0 ± 0.2	12.1 ± 0.2[c]
	18:2n-6	4.7 ± 0.8[d]	6.6 ± 0.1	8.7 ± 0.3	14.3 ± 0.4[c,d]
Platelet PL	18:1	14.8 ± 0.9[d]	17.2 ± 0.3	8.3 ± 0.4	10.1 ± 0.5[c]
	18:2n-6	3.2 ± 0.1[d]	2.9 ± 0.1	5.8 ± 0.4	8.7 ± 0.3[c,d]
Microsome PL	18:1	13.5 ± 0.3[c,d]	10.9 ± 0.8	7.8 ± 0.5	3.6 ± 0.1[c,d]
	18:2n-6	5.4 ± 0.4[d]	4.5 ± 0.5	10.8 ± 0.5	10.6 ± 0.2[c]
Microsome PE	18:1	10.0 ± 0.3[c,d]	7.6 ± 0.4	7.0 ± 0.4	3.5 ± 0.2[c,d]
	18:2n-6	3.3 ± 0.2[c,d]	2.1 ± 0.3	6.7 ± 0.3	5.4 ± 0.2[c,d]
Microsome PC	18:1	14.9 ± 0.5[c,d]	11.7 ± 0.8	8.3 ± 0.3	3.4 ± 0.1[c,d]
	18:2n-6	6.6 ± 0.6[d]	4.7 ± 0.6	11.7 ± 0.6	11.3 ± 0.3[c]
Heart PL	18:1	13.6 ± 0.3[c,d]	11.3 ± 0.3	8.0 ± 0.1	4.8 ± 0.2[c,d]
	18:2n-6	6.9 ± 0.4[c,d]	3.3 ± 0.2	11.1 ± 0.4	11.0 ± 0.3[c]
Brain PL	18:1	17.6 ± 0.6	17.0 ± 0.4	17.9 ± 1.1	18.9 ± 0.6
	18:2n-6	<0.2[d]	0.2 ± 0.1	0.6 ± 0.1	1.5 ± 0.1[c,d]
Aortic PL	18:1	30.2 ± 2.0	30.2 ± 2.2	31.1 ± 1.7	18.0 ± 0.9[c,d]
	18:2n-6	3.7 ± 0.5[d]	3.9 ± 0.3	8.7 ± 0.5	16.8 ± 2.0[c,d]

[a]Results are mean percent distributions ± SEM of fatty acid methyl esters measured on ten animals. See Table 3 for abbreviations. [b]See Table 1 for diet abbreviations. [c]$P < 0.05$, significance relative to O-25 group. [d]$P < 0.05$ significance relative to S-5 group.

in brain PL (<1.5%). In erythrocyte membrane, platelet, brain and aorta PL, animals fed the S-25 diets showed higher linoleic acid levels than animals fed the S-5 diet.

Arachidonic acid (20:4n-6) and long-chain n-6 PUFA values are shown in Table 5. Rats fed the S-5 diet showed higher levels of 20:4n-6 than rats fed the O-5 diet in erythrocyte membrane and platelet PL and in PL, PC, and PE from liver microsomes, and lower levels of 20:4n-6 in heart PL. Brain 20:4n-6 was unaffected by the diet.

The changes seen for n-6 PUFA with carbon chains of more than 18 carbon atoms (n-6 PUFA > C_{18}) (Table 5) were similar to those seen for 20:4n-6 except for heart PL which showed higher levels of n-6 PUFA > C_{18} in sunflower oil fed rats compared to olive oil fed rats; this was largely due to the increase in docosatetraenoic acid (22:4n-6) and docosapentaenoic acid (22:5n-6) which we found in heart PL (data not shown).

Linolenic acid (18:3n-3), the precursor of n-3 PUFA, was found at low levels (<1.5%) and was not affected by the diet (data not shown). Table 6 shows the data we obtained for 22:6n-3 and for n-3 PUFA longer than C_{18} (n-3 PUFA > C_{18}). The two profiles are similar, as 22:6n-3 is the fatty

TABLE 5

Arachidonic Acid and Long-Chain n-6 PUFA with More Than 18 Carbon Atoms in Phospholipids from Different Sources in Olive Oil or Sunflower Oil Fed Rats[a]

Sources	Fatty acids	Diets[b]			
		O-5	O-25	S-5	S-25
Erythrocyte PL	20:4n-6	22.0 ± 0.6[d]	21.9 ± 0.3	24.0 ± 0.3	21.2 ± 0.3[d]
	n-6 PUFA	27.7 ± 1.1[d]	26.5 ± 0.3	30.5 ± 0.4	26.3 ± 0.4[d]
Platelet PL	20:4n-6	19.0 ± 1.2[d]	18.0 ± 0.4	23.0 ± 0.6	18.1 ± 1.1[d]
	n-6 PUFA	25.2 ± 0.5[d]	23.9 ± 0.7	32.4 ± 1.0	27.1 ± 1.3[d]
Microsome PL	20:4n-6	25.8 ± 0.3[c,d]	30.7 ± 0.6	29.9 ± 0.4	31.8 ± 0.4[d]
	n-6 PUFA	29.6 ± 0.3[c,d]	32.7 ± 0.6	35.4 ± 0.5	37.5 ± 0.6[c]
Microsome PE	20:4n-6	27.1 ± 0.8[c,d]	32.4 ± 0.3	30.9 ± 0.6	33.1 ± 0.7
	n-6 PUFA	32.0 ± 0.3[d]	34.6 ± 0.3	38.7 ± 1.1	42.2 ± 0.6[c,d]
Microsome PC	20:4n-6	22.5 ± 1.1[c,d]	30.4 ± 1.1	28.3 ± 0.7	32.7 ± 0.5[d]
	n-6 PUFA	25.6 ± 1.3[c,d]	32.0 ± 1.0	31.8 ± 0.7	36.6 ± 0.6[c,d]
Heart PL	20:4n-6	26.9 ± 0.3[c,d]	29.6 ± 0.5	24.9 ± 0.2	24.8 ± 0.3[c]
	n-6 PUFA	32.8 ± 0.4[c,d]	34.8 ± 0.4	37.4 ± 0.4	39.7 ± 0.4[c,d]
Brain PL	20:4n-6	11.3 ± 0.5	11.1 ± 0.3	11.2 ± 0.5	10.9 ± 0.4
	n-6 PUFA	18.9 ± 0.6	18.0 ± 0.5	18.4 ± 1.3	16.6 ± 0.5
Aortic PL	20:4n-6	10.2 ± 0.8	12.8 ± 0.8	8.2 ± 1.0	13.2 ± 0.9[d]
	n-6 PUFA	13.8 ± 1.0	17.4 ± 1.2	10.7 ± 1.2	19.8 ± 1.1[d]

[a]Results are mean percent distributions ± SEM of fatty acid methyl esters measured on ten animals. PUFA, polyunsaturated fatty acids. See Table 3 for other abbreviations. [b]See Table 1 for diet abbreviations. [c]$P < 0.05$, significance relative to O-25 group. [d]$P < 0.05$ significance relative to S-5 group.

TABLE 6

Docosahexaenoic Acid and Long-Chain n-3 PUFA with More Than 18 Carbon Atoms in Phospholipids from Different Sources in Olive Oil or Sunflower Oil Fed Rats[a]

Sources	Fatty acids	Diets[b]			
		O-5	O-25	S-5	S-25
Erythrocyte PL	22:6n-3	1.4 ± 0.2[d]	1.9 ± 0.3	0.5 ± 0.1	0.4 ± 0.1[c]
	n-3 PUFA	2.3 ± 0.3[d]	2.9 ± 0.5	0.9 ± 0.3	0.6 ± 0.1[c]
Platelet PL	22:6n-3	<0.2	<0.2	<0.2	<0.2
	n-3 PUFA	1.8 ± 0.5	2.7 ± 0.3	1.3 ± 0.5	1.0 ± 0.2[c]
Microsome PL	22:6n-3	4.1 ± 0.1[c,d]	5.5 ± 0.3	1.2 ± 0.1	0.9 ± 0.0[c]
	n-3 PUFA	4.6 ± 0.1[c,d]	5.9 ± 0.4	1.3 ± 0.1	0.9 ± 0.0[c]
Microsome PE	22:6n-3	6.1 ± 0.2[c,d]	8.4 ± 0.4	1.9 ± 0.1	1.5 ± 0.1[c]
	n-3 PUFA	7.0 ± 0.3[c,d]	9.2 ± 0.3	2.2 ± 0.1	1.7 ± 0.1[c]
Microsome PC	22:6n-3	2.3 ± 0.2[c,d]	3.8 ± 0.2	0.7 ± 0.0	0.6 ± 0.0[c]
	n-3 PUFA	2.7 ± 0.2[c,d]	4.4 ± 0.2	0.9 ± 0.1	0.8 ± 0.1[c]
Heart PL	22:6n-3	4.4 ± 0.2[c,d]	8.2 ± 0.3	1.8 ± 0.1	1.7 ± 0.1[c]
	n-3 PUFA	5.0 ± 0.2[c,d]	9.2 ± 0.3	2.2 ± 0.1	2.1 ± 0.1[c]
Brain PL	22:6n-3	14.0 ± 0.5[d]	14.4 ± 0.3	9.5 ± 1.1	7.7 ± 0.8[c]
	n-3 PUFA	14.0 ± 0.5[d]	14.4 ± 0.3	9.5 ± 1.1	7.7 ± 0.8[c]
Aortic PL	22:6n-3	0.5 ± 0.3[d]	1.1 ± 0.7	<0.2	<0.2[c]
	n-3 PUFA	2.5 ± 0.3[d]	4.3 ± 0.6	0.6 ± 0.1	1.2 ± 0.3[c]

[a]Results are mean percent distributions ± SEM of fatty acid methyl esters measured on ten animals. See Table 3 for abbreviations. [b]See Table 1 for diet abbreviations. [c]$P < 0.05$, significance relative to O-25 group. [d]$P < 0.05$ significance relative to S-5 group.

acid contributing to the greatest extent to total PUFA. These fatty acids were found at higher levels in olive oil fed animals (5 and 25% fat diets), with the levels being always higher in the 25% olive oil fed group.

DISCUSSION

The aim of the present study was to evaluate the effect of an olive oil diet, rich in monounsaturated fatty acids, on the fatty acid compositions of different tissue PL, with a sunflower oil diet serving as reference diet.

Animals fed the olive oil diets showed the lowest total saturated fatty acid levels. These results are similar to those previously seen by our group in plasma and erythrocytes from rats fed olive oil diets as compared to those in corn oil fed rats (7); others have also reported this effect in endothelial cell cultures supplemented with oleic acid (12).

Oleic acid levels were higher in rats fed olive oil diets than in rats fed sunflower oil, except for brain PL in which similar levels were seen. This could be attributed to the high 18:1 content of the diet as well as to an increase in Δ9 desaturase activity due to olive oil feeding as we have previously described (8). It is also relevant to point out that platelets, which are deprived of desaturating capacity (31,32), showed a higher oleic acid content in high-fat fed than in low-fat fed rats, consistent with the increased intake of this fatty acid and the absence of Δ9 desaturase activity.

Linoleic acid was elevated in the two groups fed the sunflower oil diets corresponding with its high levels in the diet. Linoleic acid levels in brain were very low as has been described by others (33,34) and consistent with the effectiveness of its desaturation by this organ (33).

Arachidonic acid levels of PL are normally dependent upon the desaturating capacity of the tissue and the mode of its incorporation into cell PL (35,36). In humans, 20:4n-6 levels are greatly dependent on the diet, as Δ6 desaturation is slow and rate-limiting. In fact, under certain pathologic conditions, in which Δ6 desaturation is affected, arachidonic acid must be supplemented to the diet (37,38). In certain cell types, including erythrocytes and platelets, due to a lack in desaturating capacity, arachidonic acid levels decreased as the percentage of fat increased in the diet. By contrast, tissues with regulated desaturase activity incorporated less linoleic acid into PL and more arachidonic acid as the percentage of fat increased in the diet. In brain, the elevated levels of n-6 PUFA > C_{18} in PL, together with the low levels of linoleic acid, may indicate that dietary 18:2n-6 taken up by the brain is rapidly desaturated and elongated to long-chain PUFA. Animals fed sunflower oil diets incorporated more n-6 PUFA > C_{18} into PL of cells with desaturating capacity than did animals fed olive oil diets.

Fatty acids of the n-3 series were always elevated in olive oil-fed rats compared to sunflower oil-fed rats. The reason for this increase could be the competition between linoleic acid and α-linolenic acid for desaturases (35) which would depend on the fatty acid ratio of the diet. When the 18:2n-6/18:3n-3 ratio decreases, desaturation of 18:3n-3 is favored. In sunflower oil, the ratio of 18:2n-6/18:3n-3 is greater than in olive oil because of the high content in linoleate in the former, resulting in lower levels of long-chain n-3 PUFA found in rats fed sunflower oil as compared to olive oil fed animals.

Nevertheless, the 18:2n-6/18:3n-3 ratio might not be the only factor contributing to this increase in n-3 fatty acids, as the fat percentage in the diet also induces changes in n-3 PUFA content; rats fed the O-25 diet had, in general, higher levels of n-3 PUFA in their PL than rats fed the O-5 diet, whereas the situation in S-5 and S-25 fed rats was reversed.

The results in regard to n-3 fatty acids are of great potential interest. We have recently obtained data (22) which indicated that olive oil diets alter eicosanoid metabolism and diminish TXA_2 formation; availability of the direct precursor is an important factor in the regulation of the biosynthesis of eicosanoids in animal tissues, and precursor availability is greatly dependent on the ratio of the fatty acids in diet (39). In addition, experimental evidence indicates that n-3 fatty acids are essential for normal functional development of the retina and brain, particularly in premature infants (40–42); in various animal models, n-3 fatty acids have been shown to decrease the number and size of tumors and to increase the time elapsed before reappearance of tumors (43,44). Also, n-3 fatty acids can decrease the tendency to thrombus formation (45) and lower LDL-cholesterol (4,5), thus having potential beneficial effects in respect to coronary heart disease.

Over the past hundred years, Western diet has suffered absolute and relative changes in n-6/n-3 fatty acid ratios. There has been an increased consumption of animal fat with an imbalance in n-6/n-3 ratios as Western diets have become deficient in n-3 fatty acids (39). Appropriate n-6/n-3 ratios need to be considered when making dietary recommendations, as their balance is important in regulating homeostasis and normal development. Our data also suggest that an adequate intake of olive oil should prove beneficial in the prevention of coronary heart disease.

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Dietary ω3 Fatty Acids and Cholesterol Modify Enterocyte Microsomal Membrane Phospholipids, Cholesterol Content and Phospholipid Enzyme Activities in Diabetic Rats

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Diabetes-associated changes in intestinal uptake of nutrients are modified by isocaloric variations in the type of dietary lipids, and are associated with alterations in the phospholipid and fatty acyl content of the intestinal brush border membrane. The present study was designed to test the hypothesis that diet- and diabetes-associated changes in enterocyte microsomal membrane phospholipids are due to variations in the activity of two phospholipid metabolizing enzymes, 1,2-diacylglycerol:CDPcholine cholinephosphotransferase (CPT) and phosphatidylethanolamine methyltransferase (PEMT). Adult female Wistar rats were fed one of four semisynthetic diets—beef tallow low in cholesterol (BT), beef tallow high in cholesterol (BTC), fish oil low in cholesterol (FO) or fish oil high in cholesterol. In half of the animals, diabetes mellitus was produced by injection of streptozotocin. Jejunal and ileal enterocyte microsomes (EMM) were isolated and analyzed for cholesterol and phospholipids, as well as for CPT and PEMT activities. In control animals, feeding FO reduced EMM total phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol. Feeding FO resulted in a greater than 95% reduction in the activity of CPT. Diabetes was associated with increased jejunal EMM total phospholipids including sphingomyelin (SM) and PE, without associated changes in CPT or PEMT. Dietary cholesterol supplementation did not affect EMM total cholesterol or phospholipid composition in control rats fed BT or FO, but was associated with an increase in EMM cholesterol in diabetic rats fed BT or FO. A decrease in total phospholipids due to a decline in SM, PC and PE in diabetic rats fed FO was not associated with changes in the activities of CPT or PEMT in EMM. Thus (i) enterocyte microsomal membrane cholesterol and phospholipid contents are influenced by diabetes, dietary cholesterol and the type of fatty acid in the diet, and (ii) changes in phospholipid composition are not fully explained by alterations in the activities of CPT and PEMT.

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The absorption of nutrients is increased in streptozotocin-induced diabetes in the rat (1–11), which is associated with an increase in phospholipid content of the intestinal brush border membrane (BBM) (12,13). Diabetes-associated changes in BBM phospholipid composition were also observed, notably, a large increase in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (13). The presence of cholesterol or the fatty acid composition of the diet (saturated beef tallow or polyunsaturated ω3 fatty acids of fish oil) influences intestinal uptake of D-glucose and lipids (14,15), and also modifies BBM phospholipid and cholesterol levels (15,16). The mechanisms which control the phospholipid composition of the BBM have not yet been elucidated.

PC represents the major BBM phospholipid comprising approximately 40–45% of total BBM phospholipids (13). *De novo* PC synthesis has been described in the intestine (17–20), and a high fat diet is known to increase the activity of intestinal 1,2-diacylglycerol:CDPcholine choline phosphotransferase (CPT), the enzyme catalyzing the final step in the synthesis of PC (21). Diabetes has been shown to increase CPT activity in rat liver microsomes by 92% over that in control animals (22). The effect of diabetes or of altering the quality of the dietary fat on the activity of the rate-limiting enzyme of enterocyte *de novo* PC synthesis, CTP:phosphocholine cytidylyltransferase is not known, although changes in CPT activity have been reported to parallel those observed in CTP:phosphocholine cytidylyltransferase activity (23).

In rat liver microsomes, phosphatidylethanolamine methyltransferase (PEMT) activity was shown to be reduced by almost 50% in diabetic as compared to control rats (22). PEMT catalyzes the conversion of PE to PC with *S*-adenosylmethionine serving as the methyl donor. PEMT activity has also been observed in the BBM of the small intestine (24), although alterations that may result from diabetes or diet have not been reported.

BBM phospholipids are synthesized at the enterocyte microsomal membrane (EMM), specifically at the endoplasmic reticulum (17). The activity of PEMT or CPT in EMM may be influenced by diet and/or diabetes, thereby resulting in alterations in EMM and, subsequently, in BBM phospholipid composition. The trafficking of lipids to the BBM after synthesis in the EMM is not well understood. The BBM PC/PE ratio may also be altered by PEMT activity present in BBM.

The present study will therefore examine the effect of diet and diabetes on EMM lipid composition and will focus on two enzymes involved in the synthesis of PC. We hypothesize (i) that changes in dietary ω3 fatty acids or cholesterol affect cholesterol levels and the amounts and types of phospholipids in EMM of control and diabetic rats, and (ii) that these changes are the result of altered CPT and PEMT activities in the EMM.

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Abbreviations: BBM, brush border membrane; BT, beef tallow with low cholesterol diet; BTC, beef tallow with high cholesterol diet; CPT, 1,2-diacylglycerol:CDPcholine cholinephosphotransferase; EMM, enterocyte microsomal membranes; FO, fish oil with low cholesterol diet; FOC, fish oil with high cholesterol diet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; PI, phosphatidylinositol; SM, sphingomyelin.

METHODS AND MATERIALS

Animals and diets. The guiding principles in the care and use of laboratory animals, approved by the Canadian Federation of Biological Societies, and reviewed by the Animal Policy and Welfare Committee at the University of Alberta (Edmonton, Alberta, Canada), were observed in the conduct of this study. Female Wistar rats weighing 200–250 g were divided into two groups. The first group was rendered hyperglycemic by the intravenous injection of streptozotocin (65 mg/kg body weight). The second group was injected with saline and served as nondiabetic control. Both groups were initially fed a standard Purina® rat chow (Ralston Purina, St. Louis, MO) diet for two weeks. Then the control and diabetic groups were subdivided further into four groups of 20 rats each, and were fed one of four semisynthetic diets for two weeks—beef tallow with low (0.06%) cholesterol (BT), beef tallow with high (1%) cholesterol (BTC), fish oil with low (0.06%) cholesterol (FO), or fish oil with high (1%) cholesterol (FOC). These semisynthetic diets were nutritionally adequate, meeting known essential nutrient requirements. The composition of the diets is detailed in Tables 1 and 2.

Food intake was monitored every two to three days. Animals were weighed weekly. Blood glucose concentrations were determined one week post-injection and at the time of sacrifice. Animals were allowed access to food and water *ad libitum* until they were killed. Diet composition, food intake, weight gain and blood glucose levels have been reported (16).

Membrane preparation and analysis. EMM were prepared by a modification of the methods described by Lindeskog *et al.* (25). Intestinal mucosal scrapings were homogenized in a buffer (containing 0.25 M sucrose, 0.1 M KH_2PO_4, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 66.7 mM NaF and 0.2% trypsin inhibitor), and were centrifuged for 10 min at 800 × *g* to remove cellular debris. The supernatant was centrifuged for 30 min at 15,000 × *g* to remove mitochondrial membranes. The resultant supernatant was then centrifuged for 30 min at 48,000 × *g* to remove brush border and basolateral membranes. The final supernatant was centrifuged for 60 min at 105,000 × *g* to pellet the microsomal membranes. Glucose-6-phosphatase activity was determined to assess the purity of the microsomal preparation which was enriched approximately tenfold over homogenate (26).

Lipids of the isolated EMM were extracted immediately after preparation according to a modification of the method of Bowyer and King (27) and Folch *et al.* (28). Aliquots of the chloroform layer were removed for determining free and esterified cholesterol (29,30) and total phospholipid (31), as well as phospholipid composition (32,33).

Intestinal CPT activity was determined according to the method of Hoffman *et al.* (22). PEMT activity was assayed according to the methods of Dudeja and Brasitus (24) and Fonlupt *et al.* (34).

Expression of data. All data are expressed as mean ± SEM. Each of the eight groups consisted of 20 rats, and each sample represented material pooled from two animals, such that each group provided ten samples. The results were tested statistically by analysis of variance and by Student Neuman-Keul multiple range test.

TABLE 1

Macronutrient Composition of the Semisynthetic Diets[a]

Diet composition	g/kg
Casein	270.00
Starch	200.00
Glucose	207.65
Cellulose	50.00
Vitamin mix	10.00
Mineral mix	50.85
Choline	2.75
Inositol	6.25
L-methionine	2.50
Fat	200.00

[a]All diets were nutritionally adequate for 18:2 and 18:3. BT (beef tallow) contains 16:0 and 18:0 prepared with 18% beef tallow plus 2% safflower oil. FO (fish oil) contains 20:5ω3 and 22:6ω3 prepared with 10% fish oil plus 8% beef tallow plus 2% safflower oil. BTC (beef tallow plus cholesterol) and FOC (fish oil plus cholesterol) were prepared as above with 1% cholesterol added to the diets. Fish oil contains cholesterol (0.6% w/w), and therefore no additional cholesterol is added to the FOC diet. Thus, the final cholesterol content of FO and FOC diets was 0.06% (w/w) and 2.06% (w/w), respectively.

TABLE 2

Fatty Acid Composition of Experimental Diets[a]

Fatty acid	BT	FO
	(g/kg total diet)	
14:0	3.8	5.6
15:0	0.6	0.4
16:0	27.5	17.9
16:1ω7	0.6	6.3
17:0	2.1	0.9
18:0	52.3	25.2
18:1ω9	5.3	7.1
18:1ω7	0.1	1.3
18:2ω6	6.9	7.6
18:3ω3		3.2
20:5ω3		15.1
22:5ω3		1.3
22:6ω3		5.6

[a]Abbreviations as in Table 1.

RESULTS

EMM lipid composition. In the jejunal EMM of control rats, feeding fish oil (FO or FOC) was associated with a 42–50% decline in total phospholipid content, no change in the cholesterol content and a resultant 46–48% decline in phospholipid/cholesterol ratio as compared to animals fed beef tallow (BT or BTC) (Table 3). In diabetic rats, jejunal EMM total phospholipid content was lowered 43% and cholesterol content was unchanged; thus the phospholipid/cholesterol ratio was also lower when animals were fed FOC compared to animals fed

TABLE 3

Effect of Dietary ω3 Fatty Acids and Cholesterol on Jejunal Enterocyte Microsomal Membrane Lipid Composition in Control and Diabetic Rats[a]

Lipid (nmol/mg protein)	Control				Diabetic			
	BT	BTC	FO	FOC	BT	BTC	FO	FOC
Total phospholipids	278 ± 19	274 ± 17	160 ± 16[b]	137 ± 11[b]	324 ± 18	361 ± 15[d]	285 ± 19[d]	206 ± 18[b,c,d]
Cholesterol								
Total	91 ± 6	111 ± 7	95 ± 4	111 ± 6	101 ± 6	146 ± 7[c,d]	92 ± 5	135 ± 8[c,d]
Free	88 ± 6	101 ± 3	85 ± 5	98 ± 5	92 ± 6	125 ± 6[c]	10 ± 3	23 ± 2
Esters	4 ± 1	15 ± 4	10 ± 4	13 ± 5	9 ± 2	21 ± 3	10 ± 3	23 ± 2
Phospholipid/cholesterol	3.1 ± 0.2	2.5 ± 0.2[c]	1.7 ± 0.1[b]	1.3 ± 0.1[b]	3.3 ± 0.2	2.3 ± 0.2[c]	3.3 ± 0.3[d]	1.6 ± 0.1[b,c]

[a]The composition of the diets is given in Tables 1 and 2. For abbreviations, see Table 1. [b]$P < 0.05$, fish oil vs. beef. [c]$P < 0.05$, cholesterol supplementation. [d]$P < 0.05$, diabetic vs. control.

BTC. Cholesterol supplementation did not significantly alter the jejunal EMM phospholipid and cholesterol levels of control animals fed beef tallow or fish oil, although small changes resulted in a 20% decline in the phospholipid/cholesterol ratio in animals fed beef tallow (BTC vs. BT). By contrast, in the jejunal EMM of diabetic animals, cholesterol supplementation decreased the phospholipid content by 28% in animals fed fish oil, and increased the cholesterol content by 46% in animals fed either beef tallow or fish oil, which resulted in a phospholipid/cholesterol ratio that was reduced by 30 and 52%, respectively. Diabetes was associated with an increase in jejunal EMM total phospholipid content in animals fed BTC (32%), FO (78%) and FOC (50%), and an increase in total cholesterol content in animals fed BTC (32%) and FOC (22%) as compared with controls. The phospholipid/cholesterol ratio was not significantly altered in diabetes except for the twofold increase in the diabetic compared with the control animals fed FO.

Ileal EMM phospholipid and cholesterol content was unaffected by fish oil feeding in both control and in diabetic animals (Table 4). Although ileal EMM phospholipid content and the phospholipid/cholesterol ratio were unchanged, the cholesterol content was significantly increased (by 36%) when control animals fed beef tallow were supplemented with cholesterol. In diabetic animals fed fish oil, cholesterol supplementation did not significantly affect phospholipid content, but increased ileal EMM cholesterol content 42% and reduced the phospholipid/cholesterol ratio by 45%. Cholesterol supplementation did not alter the ileal EMM phospholipid and cholesterol content of control animals fed fish oil, or diabetic animals fed beef tallow. Although diabetes was associated with a 19% increase in ileal EMM cholesterol content in animals fed FOC, the total phospholipid levels and the phospholipid/cholesterol ratio remained unchanged when compared with controls fed FOC. Diabetes did not significantly alter the ileal EMM phospholipid and cholesterol content of animals fed BT, BTC or FO as compared with controls.

EMM phospholipid composition. Feeding FO was associated with a decline in sphingomyelin (SM), PC and PE in jejunal EMM of control animals, as compared with those fed BT, by 37, 54 and 35%, respectively. Feeding FOC was associated with a 53% reduction in PE and a 63% reduction in phosphatidylinositol (PI) compared with feeding BTC (Table 5). In the jejunal EMM of diabetic animals, feeding FO was also associated with a 57% decrease in SM when compared with feeding BT, whereas feeding FOC was associated with a decline in SM (56%) and PE (46%) when compared with feeding BTC. Cholesterol supplementation did not alter the phospholipid composition of jejunal or ileal EMM in control animals fed either beef tallow or fish oil. In the jejunal EMM of diabetic animals, cholesterol supplementation was associated with an increase in SM (24% and PE

TABLE 4

Effect of Dietary ω3 Fatty Acids and Cholesterol on Ileal Enterocyte Microsomal Membrane Lipid Composition in Control and Diabetic Rats[a]

Lipid (nmol/mg protein)	Control				Diabetic			
	BT	BTC	FO	FOC	BT	BTC	FO	FOC
Total phospholipids	203 ± 21	198 ± 15	170 ± 10	152 ± 11	226 ± 16	228 ± 13	210 ± 9	165 ± 17
Cholesterol								
Total	98 ± 7	133 ± 12[c]	104 ± 5	123 ± 7	119 ± 9	136 ± 8	110 ± 6	156 ± 7[c,d]
Free	94 ± 7	109 ± 8	97 ± 6	109 ± 5	104 ± 5	110 ± 6	99 ± 5	123 ± 6
Esters	6 ± 2	24 ± 10	9 ± 3	15 ± 3	15 ± 5	26 ± 4	11 ± 3	33 ± 4[c,d]
Phospholipid/cholesterol	2.1 ± 0.2	1.6 ± 0.1	1.7 ± 0.2	1.2 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	1.1 ± 0.1[c]

[a]The composition of the diets and the abbreviations are given in Tables 1 and 2. [b]$P < 0.05$, fish oil vs. beef. [c]$P < 0.05$, cholesterol supplementation. [d]$P < 0.05$, diabetic vs. control.

TABLE 5

Effect of Dietary ω3 Fatty Acids and Cholesterol on Jejunal Enterocyte Microsomal Membrane (EMM) Phospholipid Composition in Control and Diabetic Rats[a]

	Control				Diabetic			
	BT	BTC	FO	FOC	BT	BTC	FO	FOC
Sphingomyelin (SM)								
%	15.96 ± 2.07	17.52 ± 1.73	19.44 ± 2.21	21.52 ± 2.22	19.19 ± 2.08	20.71 ± 1.53	9.12 ± 1.07[b,d]	16.02 ± 1.32[c]
nmol/mg protein	46.20 ± 6.32	47.68 ± 4.97	28.79 ± 2.00[b]	28.47 ± 3.05	59.19 ± 4.95[d]	73.67 ± 6.05[c,d]	25.16 ± 1.99[b]	32.10 ± 1.97[b]
Phosphatidylcholine (PC)								
%	24.87 ± 2.87	19.24 ± 1.84	20.90 ± 1.70	21.68 ± 2.41	24.10 ± 2.10	17.17 ± 1.83	30.72 ± 2.13[d]	24.67 ± 2.08
nmol/mg protein	76.10 ± 12.90	52.34 ± 5.49	34.78 ± 5.29[b]	30.34 ± 4.17	76.81 ± 8.02	62.13 ± 8.03	92.98 ± 11.18[d]	54.24 ± 8.20[c]
Phosphatidylserine								
%	2.29 ± 0.60	2.82 ± 1.12	0.70 ± 0.30	1.18 ± 0.42	1.89 ± 0.46	2.18 ± 0.49	1.44 ± 0.33	1.26 ± 0.70
nmol/mg protein	7.18 ± 3.31	6.96 ± 2.36	1.04 ± 0.43	1.54 ± 0.56	6.11 ± 1.47	7.75 ± 1.77	3.72 ± 0.62	2.62 ± 1.27
Phosphatidylethanolamine (PE)								
%	46.00 ± 2.53	50.73 ± 1.61	48.45 ± 1.52	47.11 ± 2.14	45.49 ± 2.07	52.73 ± 1.84	48.40 ± 1.84	47.93 ± 1.73
nmol/mg protein	119.8 ± 15.28	139.9 ± 11.27	77.48 ± 7.76[b]	65.09 ± 7.09[b]	156.6 ± 14.74	187.0 ± 10.18[c,d]	140.6 ± 851[d]	100.5 ± 9.63[b,c,d]
Phosphatidic acid								
%	0.51 ± 0.25	0.82 ± 0.40	1.04 ± 0.56	1.43 ± 0.59	0.26 ± 0.13	0.11 ± 0.08	0.70 ± 0.27	0.89 ± 0.62
nmol/mg protein	1.62 ± 0.97	2.14 ± 1.02	1.17 ± 0.57	1.52 ± 0.54	0.77 ± 0.36	0.33 ± 0.22	1.67 ± 0.66	1.36 ± 0.99
Phosphatidylinositol								
%	10.28 ± 0.95	8.52 ± 0.72	9.33 ± 2.23	6.60 ± 09.74	7.53 ± 0.59	6.72 ± 0.47	9.24 ± 0.72	7.55 ± 0.72
nmol/mg protein	23.63 ± 4.73	23.64 ± 2.80	16.60 ± 4.62	8.78 ± 1.01[b]	23.70 ± 2.08	24.23 ± 2.39	28.08 ± 3.04	16.41 ± 2.89
Choline/amine	0.89 ± 0.11	0.69 ± 0.04	0.83 ± 0.05	0.93 ± 0.08	0.98 ± 0.08	0.71 ± 0.06	0.83 ± 0.08	0.88 ± 0.07
PC/PE	0.58 ± 0.10	0.39 ± 0.05	0.43 ± 0.04	0.48 ± 0.06	0.55 ± 0.06	0.33 ± 0.05	0.66 ± 0.07	0.53 ± 0.06
SM/PC	0.73 ± 0.13	1.06 ± 0.19	1.06 ± 0.21	1.21 ± 0.24	0.92 ± 0.18	1.31 ± 0.17	0.34 ± 0.07[d]	0.72 ± 0.10

[a]The composition of the diets and the abbreviations are given in Tables 1 and 2. [b]$P < 0.05$, fish oil vs. beef. [c]$P < 0.05$, cholesterol supplementation. [d]$P < 0.05$, diabetic vs. control.

TABLE 6

Effect of Dietary ω3 Fatty Acids and Cholesterol on Ileal Enterocyte Microsomal Membrane Phospholipid Composition in Control and Diabetic Rats[a]

	Control				Diabetic			
	BT	BTC	FO	FOC	BT	BTC	FO	FOC
Sphingomyelin (SM)								
%	24.90 ± 1.55	27.55 ± 2.34	25.72 ± 2.99	27.21 ± 2.16	26.75 ± 2.57	29.72 ± 1.95	18.65 ± 3.21	25.34 ± 2.01
nmol/mg protein	50.60 ± 5.28	53.19 ± 4.66	42.94 ± 6.11	40.26 ± 4.04	55.63 ± 3.63	69.26 ± 5.35	39.19 ± 6.26	45.54 ± 2.84[b]
Phosphatidylcholine (PC)								
%	22.43 ± 3.70	15.13 ± 1.51	18.11 ± 2.01	17.90 ± 2.27	15.91 ± 1.73	15.41 ± 1.55	23.16 ± 1.54	24.40 ± 3.37
nmol/mg protein	50.13 ± 10.63	31.19 ± 5.22	30.70 ± 4.41	26.87 ± 3.72	33.89 ± 3.78	36.00 ± 4.01	50.05 ± 3.79	46.60 ± 8.31
Phosphatidylserine								
%	2.42 ± 0.78	1.30 ± 0.43	1.70 ± 0.50	2.23 ± 0.73	3.35 ± 0.50	2.56 ± 0.48	2.78 ± 0.38	2.66 ± 0.58
nmol/mg protein	4.67 ± 1.50	2.67 ± 0.88	2.94 ± 0.89	3.10 ± 1.02	7.46 ± 1.43	5.98 ± 1.01	6.18 ± 0.89	4.86 ± 1.09
Phosphatidylethanolamine (PE)								
%	37.01 ± 2.70	44.22 ± 1.58	46.60 ± 3.01	44.16 ± 2.58	46.22 ± 2.88	43.82 ± 1.96	46.29 ± 2.76	37.59 ± 2.42
nmol/mg protein	78.47 ± 9.64	86.63 ± 5.93	76.34 ± 5.35	66.63 ± 8.13	102.3 ± 12.07	103.5 ± 9.39	100.9 ± 7.57	68.27 ± 4.44
Phosphatidic acid								
%	1.71 ± 0.69	1.83 ± 0.64	1.90 ± 0.71	1.34 ± 1.04	0.59 ± 0.29	0.74 ± 0.27	1.28 ± 0.64	1.69 ± 0.52
nmol/mg protein	3.45 ± 1.72	4.26 ± 1.92	3.15 ± 1.22	1.58 ± 1.17	1.04 ± 0.47	1.58 ± 0.52	2.50 ± 1.15	2.64 ± 0.97
Phosphatidylinositol								
%	10.20 ± 2.00	7.65 ± 1.19	5.78 ± 1.50	5.37 ± 1.18	6.69 ± 0.57	6.49 ± 0.86	7.49 ± 1.14	8.17 ± 0.71
nmol/mg protein	19.89 ± 3.64	14.49 ± 2.08	9.15 ± 2.36[b]	9.83 ± 2.17	14.02 ± 1.02	15.00 ± 1.94	15.10 ± 2.99	15.26 ± 1.90
Choline/amine	1.26 ± 0.15	0.96 ± 0.08	0.97 ± 0.13	1.00 ± 0.09	0.93 ± 0.12	1.02 ± 0.12	0.97 ± 0.17	1.33 ± 0.16
PC/PE	0.64 ± 0.12	0.35 ± 0.04	0.41 ± 0.06	0.42 ± 0.06	0.36 ± 0.05	0.37 ± 0.06	0.54 ± 0.07	0.71 ± 0.13[b]
SM/PC	1.44 ± 0.28	2.11 ± 0.36	1.55 ± 0.23	2.03 ± 0.55	1.90 ± 0.28	2.05 ± 0.21	0.90 ± 0.22	1.28 ± 0.24

[a]The composition of the diets and the abbreviations are given in Tables 1 and 2. [b]$P < 0.05$, fish oil vs. beef.

(27%) in animals fed beef tallow, whereas in diabetic animals fed fish oil, cholesterol supplementation was associated with a reduction in PC (42%) and PE (29%). Diabetes was associated with an increase in jejunal EMM SM (28–55%) and PE (22–34%) in animals fed beef tallow (BT or BTC). In animals fed FO, diabetes was associated with an increase in jejunal EMM PC (167%) and PE (81%) with a resultant 68% reduction in SM/PC ratio, and in animals fed FOC only PE was increased (94%).

In the ileal EMM of control animals, feeding FOC did not alter phospholipid composition compared with feeding BTC, although feeding FO was associated with a 54% reduction in PI compared with feeding BT (Table 6). In diabetic rats, feeding FO did not alter the ileal EMM phospholipid composition compared with diabetic rats fed BT, while feeding FOC was associated with a decline in SM (34%) and a 92% increase in PC/PE ratio compared with feeding BTC. Ileal EMM phospholipid composition was unaffected by cholesterol supplementation in diabetic animals fed either beef tallow or fish oil.

CPT and PEMT activities. Feeding fish oil (FO or FOC), when compared with feeding beef tallow (BT or BTC), was associated with a marked decline in jejunal EMM CPT activity of control (94–95%) and diabetic rats (74–82%), whereas the decline in ileal CPT activity in control (63–70%) and in diabetic (74–76%) rats did not reach statistical significance ($P < 0.05$) (Table 7). CPT was not significantly affected by adding cholesterol to the diet, or by diabetes. The general decline in jejunal and ileal EMM PEMT activity observed with diabetes in the absence of dietary cholesterol did not reach statistical significane. PEMT activity was not significantly affected by dietary ω3 fatty acids or by cholesterol supplementation.

DISCUSSION

Feeding a polyunsaturated fatty acid diet may reduce the enhanced uptake of glucose and lipids in diabetic rats (16,35,36). Alterations in the lipid composition and properties of the BBM have been postulated to explain this decline in nutrient absorption (16,37). Diabetes is associated with an increase in BBM total phospholipids due to an increase in PC and PE (13). This diabetes-associated increase in BBM PC and PE, and the fish oil-associated decline in BBM PC and PE led to the hypothesis that the synthesis of phospholipids in the enterocyte is influenced by diet and diabetes. The next step was to examine the site of lipid synthesis and membrane assembly, namely the EMM.

Diabetes was found to be associated with an increase in jejunal EMM total phospholipid content in rats fed BTC, FOC and FO, with this increase being primarily due to an increase in PE content in all groups, although SM was also increased in animals fed BTC (Tables 3–6). The increase in PE was not associated with any changes in the activity of PEMT (Table 7), but may be due to changes in the enzymes involved in *de novo* PE synthesis. The increase in EMM SM levels with diabetes in animals fed beef tallow suggests that future studies should

TABLE 7

Effect of ω3 Fatty Acids and Cholesterol on Enterocyte Microsomal Membrane Phospholipid Enzyme Activity in Control and Diabetic Rats[a]

	Control				Diabetic			
	BT	BTC	FO	FOC	BT	BTC	FO	FOC
Phosphocholine transferase (nmol/mg protein/min)								
Jejunum	0.145 ± 0.027	0.104 ± 0.018	0.008 ± 0.005[b]	0.005 ± 0.002[b]	0.155 ± 0.019	0.137 ± 0.024	0.041 ± 0.011[b]	0.024 ± 0.009[b]
Ileum	0.052 ± 0.013	0.040 ± 0.009	0.019 ± 0.005	0.012 ± 0.003	0.059 ± 0.009	0.051 ± 0.016	0.021 ± 0.005	0.012 ± 0.005
Phosphatidylethanolamine methyltransferase (pmol/mg protein/min)								
Jejunum	0.042 ± 0.016	0.013 ± 0.007	0.044 ± 0.027	0.035 ± 0.021	0.014 ± 0.010	0.005 ± 0.003	0.011 ± 0.004	0.028 ± 0.017
Ileum	0.076 ± 0.017	0.046 ± 0.018	0.060 ± 0.025	0.037 ± 0.014	0.030 ± 0.012	0.021 ± 0.006	0.034 ± 0.008	0.038 ± 0.021

[a]The composition of the diets and abbreviations are given in Tables 1 and 2. [b]$P < 0.05$, fish oil vs. beef.

also consider examining the activity of sphingomyelinase. Insulin increases sphingomyelinase activity (38), and therefore diabetes may be associated with a decline in the activity of this enzyme and thereby lead to increased EMM SM levels.

The total cholesterol content of jejunal EMM was approximately 45% higher in diabetic than in control rats, but only when they were fed a high cholesterol diet (BTC or FOC). Cholesterol absorption is known to be increased in diabetes (39). Control animals appear to maintain a constant EMM cholesterol content even in the face of a high dietary content of cholesterol. This homeostatic regulation appears to fail in diabetes. The inability of the diabetic rat jejunal EMM to cope with an increased dietary load of cholesterol may be related to the known changes in cholesterol metabolism that are observed in diabetes. Intestinal *de novo* synthesis of cholesterol has been shown to be increased 2–3-fold in streptozotocin-diabetic rats (40) associated with an increase in 3-hydroxy-3-methylglutaryl-CoA reductase activity (41–43). Cholesterol feeding did not alter cholesterol synthesis in control animals, but markedly inhibited cholesterol synthesis in the diabetic small intestine (44). In control rats, feeding a diet enriched in ω3 polyunsaturated fatty acids is associated with an increase in acyl-CoA:cholesterol acyltransferase activity and is not increased further with dietary cholesterol supplementation (45,46). The mechanism by which jejunal EMM cholesterol is increased in diabetic rats on a high cholesterol diet is unknown, but may be related to the possible increased availability of dietary cholesterol for membrane synthesis resulting from exceeding the capacity of the increased lipoprotein transport from the intestine to the circulation observed with diabetes (47).

BBM phospholipid content is reduced by fish oil feeding (16), although the mechanism of this alteration is not clear. Feeding fish oil also reduced the total phospholipid content of control jejunal EMM (Table 3), which resulted in a decrease in the phospholipid-to-cholesterol ratio, with changes also occurring in individual phospholipids including SM, PC and PI (Table 5). Fish oil feeding does not appear to alter membrane cholesterol content compared to feeding beef tallow. In diabetic animals, feeding fish oil reduced the total phospholipid content of jejunal EMM of animals fed the high cholesterol diet (FOC), but not those fed the low cholesterol diet (FO). This decline in total phospholipids was a consequence of the reduction in SM and PE (Table 5). The data suggest that feeding ω3 polyunsaturated fatty acids is associated with a decrease in phospholipid synthesis.

The decrease in EMM PC observed with fish oil feeding (Table 5) led us to postulate that enterocyte microsomal PC synthesis may be affected by the CPT and the PEMT pathways. The decrease in PC observed in jejunal EMM of control rats fed FO was associated with a marked reduction in the activity of EMM CPT (Table 7), the enzyme catalyzing the last step in the PC synthesis by the CDPcholine pathway. The decline in PE in control rats fed FO or FOC, and in diabetic rats fed FOC (Table 5), cannot be explained by differences in microsomal PEMT activity (Table 7) and may involve a decrease in the *de novo* synthesis of PE. The marked reduction in EMM SM content with fish oil feeding may be due to a decline in *de novo* synthesis or an increase in sphingomyelinase activity.

Cholesterol supplementation did not alter jejunal or ileal EMM total phospholipid content or composition in control animals (Tables 3 and 5). In diabetic rats, cholesterol feeding did not alter the jejunal EMM total phospholipid content when fed beef tallow (BTC vs. BT), but was associated with a decline in total phospholipids when fed fish oil (FOC vs. FO) due to a reduction in PE. Thus, dietary cholesterol may influence the regulation of *de novo* phospholipid synthesis in the rat small intestine, as it does in rat liver (48).

Whereas the jejunal EMM was responsive to the effects of dietary fatty acids and cholesterol, and to diabetes, the ileal EMM showed relatively few changes, namely a decline in SM and an increase in PC/PE ratio with fish oil feeding in diabetes (FOC vs. BTC), an increased cholesterol content, and reduced phospholipid/cholesterol ratio with cholesterol feeding in diabetes (FOC vs. FO) (Tables 4 and 6). Except for an increase in cholesterol content with diabetes in animals fed FOC, diabetes was not associated with any change in phospholipid, cholesterol and phospholipid composition. The activities of CPT and PEMT in ileal EMM did not respond to fish oil feeding (Table 7). It is possible that the EMM are inherently different for the ileum and for the jejunum, as is the case for microsomal desaturase activities (49). Alternatively, the dietary signal to adapt is likely much stronger in the jejunum than in the ileum, and when ileal EMM composition is modified, the change is qualitatively similar to that in the jejunum. Whether the variable responses between jejunum and ileum are on the basis of qualitative or quantitative differences remains the subject of future study.

Alterations in membrane fluidity arise from changes in membrane lipid composition and may play a role in the regulation of membrane-bound enzymes (50). Diabetes was associated with a decrease in BBM fluidity and an increase in sodium-dependent D glucose transport (51,52). Hepatic desaturase activity is also influenced by hepatic microsomal membrane lipid composition (53). Diabetes and dietary lipid-associated changes in EMM cholesterol and phospholipid content may be associated with a variety of changes in EMM fluidity which, in turn, may have influenced the activity of intrinsic membrane proteins such as CPT and PEMT. However, in addition to membrane cholesterol content, phospholipid content and phospholipid composition, phospholipid fatty acid composition is also an important determinant of membrane fluidity. The decline in CPT activity observed with fish oil feeding may be related to a change in fluidity resulting from feeding a diet enriched with ω3 polyunsaturated fatty acids. Future studies may examine the effect of diabetes and diet on EMM fluidity and phospholipid fatty acid composition.

Until the actual signals for EMM adaptation in response to diabetes and dietary manipulation are identified, the mechanisms responsible for the control of mem-

brane composition and adaptation remain the subject of further investigation.

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Hematological and Lipid Changes in Newborn Piglets Fed Milk Replacer Diets Containing Vegetable Oils with Different Levels of n-3 Fatty Acids

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To test if linolenic acid (18:3n-3) from vegetable oils would affect bleeding times and platelet counts in newborns, piglets were used as a model fed milk replacer diets containing 25% (by wt) vegetable oils or oil mixtures for 28 d and compared to sow-reared piglets. The oils tested included soybean, canola, olive, high oleic sunflower (HOAS), a canola/coconut mixture and a mixture of oils mimicking canola in fatty acid composition. All piglets fed the milk replacer diets showed normal growth. Bleeding times increased after birth from 4–6 min to 7–10 min by week 4 ($P < 0.001$), and were higher in pigs fed diets containing 18:3n-3, as well as in sow-reared piglets receiving n-3 polyunsaturated fatty acids (PUFA) in the milk, as compared to diets low in 18:3n-3. Platelet numbers increased within the first week in newborn piglets from 300 to 550 $\times 10^9$/L, and remained high thereafter. Milk replacer diets, containing vegetable oils, generally showed a transient delay in the rise of platelet numbers, which was partially associated with an increased platelet volume. The oils showed differences in the length of delay, but by the third week of age, all platelet counts were >500 $\times 10^9$/L. The delay in rise in platelet counts appeared to be related to the fatty acid composition of the oil, as the effect was reproduced by a mixture of oils with a certain fatty acid profile, and disappeared upon the addition of saturated fatty acids to the vegetable oil. There were no alterations in the coagulation factors due to the dietary oils. Blood plasma, platelets and red blood cell membranes showed increased levels of 18:3n-3 and long-chain n-3 PUFA in response to dietary 18:3n-3. The level of saturated fatty acids in blood lipids was generally lower in canola and HOAS oil-fed piglets as compared to piglets fed soybean oil or reared with the sow. The results suggest that consumption of milk replacer diets containing vegetable oils rich in 18:3n-3 does not represent a bleeding risk, and that the transient lower platelet count can be counterbalanced by the addition of saturated fatty acids to the vegetable oils.

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Abbreviations: ANOVA, analysis of variance; APTT, activated partial thromboplastin time; C, cholesterol; CE, cholesteryl ester; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; HEAR, high erucic acid rapeseed; HOAS, high oleic acid sunflower; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; PC, phosphatidylcholine; PCV, packed cell volume; PE, phosphatidylethanolamine; PS, phosphatidylserine; PT, prothrombin time; PUFA, polyunsaturated fatty acids; RBC, red blood cell; SP, sphingomyelin; TAG, triacylglycerol; WBC, white blood cell.

While larger intakes of long-chain n-3 polyunsaturated fatty acids (PUFA) from fish oil in human diets have been shown to be associated with a number of favorable effects, such as reduced coronary heart disease, improved serum lipid profiles and decreased platelet aggregation (1–4), there have also been some concerns, for example with respect to prolonged bleeding times (3,5–14) and a decrease in platelet counts (3,5,6,8–10,14–17). These changes are believed to be due to the incorporation of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids into platelet phospholipids, resulting in alterations in platelet and hemostatic function.

Based on the metabolic conversion of dietary linolenic acid (18:3n-3) to 20:5n-3 and 22:6n-3 in animals and in humans (1,18,19), one might expect the same effects from vegetable oils containing 18:3n-3. A number of studies have shown that the consumption of diets containing 18:3n-3 increased the long-chain n-3 PUFA in blood lipids (20–27), but the increases were less than those seen with the inclusion of fish oils containing 20:5n-3 and 22:6n-3 (20,28). The possibility that vegetable oils containing substantial amounts of 18:3n-3 might affect bleeding times or platelet counts was recently investigated. In three studies with humans, bleeding times were either moderately increased (25,29) or remained the same (27) with the consumption of canola oil, but changes in platelet counts were not investigated. In a study on newborn piglets, a reduction in platelet count was observed after 18 d when oil/fat mixtures were fed containing about 33% canola oil or high oleic acid safflower or sunflower oil (28), but bleeding times were not measured.

The possible risk of increased bleeding and thrombocytopenia due to dietary n-3 fatty acids would be of particular concern to infants because of their greater susceptibility to injury, their higher intake of food per unit body weight and their often restricted choice in diet. The recent interest to improve the n-3 fatty acid contents of infant formulas (4,30,31) might lead to the inclusion of increased 18:3n-3 from vegetable oils to satisfy the n-3 requirement. The increased 18:3n-3 content in infant formulas needs to be evaluated in the light of the apparent risks involved.

In the present study, the risk factors of increased bleeding and decreased platelet counts were assessed in newborn piglets fed milk replacer diets containing various vegetable oils with different levels of 18:3n-3 as the sole source of fat in the diet. The pig was chosen because it is believed to be a suitable model for hematological studies in humans (32). The oils tested included soybean

oil and canola oil which, in addition to 18:3n-3, contained high levels of either linoleic acid (18:2n-6) or oleic acid (18:1n-9), respectively. Oils high in 18:1n-9 and low in 18:3n-3, i.e., high oleic acid sunflower (HOAS) oil and olive oil, were used for comparison. Sow-reared groups were included which received preformed C_{20} and C_{22} n-6 and n-3 PUFA from maternal milk. Canola oil was included in order to compare its effects with those of the other oils. Canola oil is presently excluded from infant formulas (33) in the United States, as its safety has not been conclusively documented.

MATERIALS AND METHODS

Animals and housing. Newborn Yorkshire male and female piglets were left with the sow for one day to permit consumption of colostrum. Littermates were allocated randomly to the diets so that the diets were balanced for litter and sex. Piglets were housed individually in stainless steel cages, arranged in banks of six (34), which were equipped with a common milk replacer reservoir and an automatic feed dispensing system. Animals were fed every two hours at a rate of solid intake equivalent to 7% of body weight/day. Body weights were recorded every three days and feed was adjusted accordingly.

Diets. The oils used in these studies, alone or in mixtures, included: soybean oil (CanAmera Foods Inc., Toronto, Ontario, Canada), HOAS oil and olive oil (Loblaws Ltd., Ottawa, Ontario, Canada) canola and high erucic acid rapeseed (HEAR) oils (CanAmera Foods, Inc., Altona, Manitoba, Canada), and linseed oil (gift from Dr. J.D. Jones, Ottawa, Ontario, Canada). The fatty acid composition of the test oils and oil mixtures are shown in Table 1. Small amounts of HEAR oil were added to canola oil to bring the level of erucic acid (22:1n-9) to about 2%, which is the maximum permissible level of 22:1n-9 in canola oil in the United States (33). A mixture of oils was prepared from HOAS oil, soybean oil and linseed oil to mimic the fatty acid composition of canola oil, except without 22:1n-9. Only sow milk contained long-chain n-6 and n-3 PUFA.

TABLE 1

Fatty Acid Composition of Sow Milk and Oils Used in Milk Replacer Diets

Fatty acids	Sow milk[a]	Soybean oil	HOAS[b] oil	Canola oil[c]	Mixture of oils[d]
≤14:0	2.1	0.1	0.1	0.1	0.1
16:0	23.3	10.3	3.9	5.0	6.8
16:1	4.6	0.2	0.1	0.3	0.1
18:0	6.1	3.4	4.4	2.0	4.3
18:1n-9	32.4	22.3	76.1	52.0	54.1
18:1n-7	2.8	1.4	0.8	2.6	1.3
18:2n-6	22.9	48.4	11.9	20.8	23.7
18:3n-3	1.1	7.7	0.1	7.8	7.3
20:0	0.2	0.3	0.4	0.6	0.4
20:1n-9	0.6	0.3	0.3	1.6	0.3
22:0	0.1	0.3	1.1	0.4	0.8
22:1n-9	0.1	0	0	1.6	0
24:0	0.1	0.1	0.4	0.2	0.2
24:1n-9	0.1	0	0	0.2	0

[a]Sow milk contained 1.8% 14:0, 1.6% n-6 PUFA (polyunsaturated fatty acids; 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6), and 0.7% n-3 PUFA (20:5n-3, 22:5n-3 and 22:6n-3).
[b]HOAS, high oleic acid sunflower.
[c]Mixture of canola oil (0.8%, 22:1n-9) and high erucic acid rapeseed oil (42.9%, 22:1n-9) in the ratio of 98:2.
[d]Mixture of HOAS oil, soybean oil and linseed oil in a ratio of 55:31:14.

Milk replacer diets were stored at -20°C and prepared daily to include 21% solids of which dietary oil was 25% by weight (~50% of calories) and γ-globulin was 16% (35). Formalin (40% solution) was added as a preservative at 0.1% of total milk replacer. The digestible energy content of the final milk replacer was 953 kcal/L (35).

The level of vitamin E (α-tocopherol) included was 22 IU/kg diet, or 37% more than the National Research Council (NRC) requirements given for newborn piglets (36). This level appears to be too low as assessed by liver vitamin E concentrations of vitamin E, which ranged from 1–4 ng/mg wet weight. The vitamin E in the diet was therefore increased to 100 (one canola group, Experiment I) and 150 IU/kg (all diets, Experiment II) with a corresponding increase to about 7 and 14 ng/mg wet weight of liver for Experiments I and II, respectively. Sow milk contains about 2 μg/mL α-tocopherol.

Bleeding time measurements. Bleeding measurements were taken at one day of age and every week thereafter by the same person. Piglets were placed in a harness, a small area of the shoulder was cleaned and shaved, and an incision was made using a Simplate II device (Organon Teknika, Scarborough, Ontario, Canada) which gave two cuts 5 mm in length and 1 mm deep. Every 30 s a filter paper disc was carefully placed near the cut without disturbing it. The bleeding time was recorded as the time between the incision and when the filter paper no longer stained.

Blood sampling and measurements. Blood for hematological measurements was taken by orbital sinus puncture (37), and 0.5 mL was collected in each of two tubes, one containing sodium citrate (for coagulation studies), the other ethylenediaminetetraacetic acid (EDTA, for complete blood counts) as anticoagulant. The blood was thoroughly mixed with the anticoagulants and transported immediately for analysis. Complete blood count including a platelet count and mean platelet volume (MPV) and differential were measured using a Technicon H-2 analyzer (Technicon Instrument Corp., Tarrytown, NY).

Lipid analyses. For lipid analyses, about 15 mL of blood was obtained from the jugular vein using vacutainer tubes containing EDTA. The blood was taken from three piglets/diet at time of slaughter. Whole blood was separated into plasma, platelets and red blood cell (RBC) membranes as follows: the blood was centrifuged as soon as possible at 150 × *g* for 15 min. The plasma was removed, adjusted to pH 6.5 with 1 M citric acid, and two drops of EDTA were added. The plasma was centrifuged again at 150 × *g* to remove traces of red and white cells, and then at 2500 × *g* for 15 min to remove the platelets. The plasma was stored at –70°C until analyzed for lipids. The platelets were suspended in 1 mL of buffer (20 mM

Na_2HPO_4, 130 mM NaCl and 2 mM EDTA at pH 6.5) containing inhibitors (10 µL of 4 mM iodoacetamide, 20 µL of 4 mM *N*-ethylmaleimide in 50% ethanol, and 10 µL of aprotinin containing 10,000 units/mL) and centrifuged at 2500 × *g* for 15 min. The platelets were then resuspended in 1 mL of buffer plus inhibitors and stored at –70°C. For lipid analyses, the platelets from the three piglets/diet were combined. The RBC membranes were prepared from RBC as described (38).

Platelets and RBC membranes were taken up in chloroform/methanol/water (1:2:0.8, by vol) and thoroughly mixed. More chloroform and water was then added to give a final volume ratio of 2:2:1.8 for chloroform/methanol/water (39). The chloroform layer contained the total lipids. The total lipids from serum were extracted as described (40). Lipid classes were separated by thin-layer chromatography using silica gel H coated plates (41), and observed under ultraviolet light after spraying the plates with 2′,7′-dichlorofluorescein. Fractions were scraped off, methyl heptadecanoate was added as internal standard, and the lipids were transmethylated in the presence of silica gel using anhydrous HCl/methanol (5% by wt). The resulting methyl esters and dimethyl acetals were analyzed by gas–liquid chromatography (42).

Bone marrow examination. Sections of femoral bone marrow were taken at 28 d in Experiment II. Samples were fixed in formalin, embedded in paraffin and 5-µm thick sections were stained with hematoxylin and eosin and examined by light microscopy.

Statistics. Data from each age set were examined separately using analysis of variance (ANOVA). The terms in the model for the milk replacer-fed piglets were litter and treatment. The data were reanalyzed using the 0 d data as a covariate. The inferences were very similar from this analysis, and therefore only the results from the ANOVA are presented. To examine patterns during each experiment, the data were reanalyzed using a repeated measures ANOVA, thereby allowing differences over time to be evaluated on a within-piglet basis. For none of the parameters analyzed was diet × age interaction significant ($P > 0.05$). The litter effect was not significant ($P > 0.05$) for the parameters considered here, and, therefore, the hematological data and the lipid and fatty acid composition data (three piglets chosen at random from diet groups in Experiment II) were examined using a one-way ANOVA with treatments as the only term in the model. Results were considered to be statistically significant whenever $P < 0.05$. Data presented in Tables 2 and 3 are least square means because some values are missing; mean significant levels were based on the least significant difference test.

RESULTS

Diets and growth. The fatty acid composition of the dietary oils used in the studies is shown in Table 1. There were no significant differences in growth among piglets fed the different artificial milk replacers, and, for that reason, the results of the four diets in Experiment II were combined in Figure 1. The body weights of piglets left nursing the sow were significantly greater during the first week compared to piglets fed the milk replacer diets, but by the fourth week this was reversed. When milk replacers were fed at 7% of body weight, there were no significant differences in milk consumption among the different diets (Fig. 1).

Bleeding times. The bleeding times of one-day-old piglets ranged from 4–6 min and increased significantly ($P < 0.001$) with age on all milk replacer diets (Fig. 2). Generally, oils containing 18:3n-3 (soybean oil, canola oil, mixture of oils) gave longer bleeding times than oils low in 18:3n-3 (HOAS oil) (Experiment II, 28 d). There were no significant differences in bleeding times between canola and soybean oil-fed piglets and between the mixture of oils (similar to canola oil in fatty acid composition) and canola oil (Experiment II).

Platelets. The platelet results from two additional unpublished studies (III and IV) are included in Table 2. One-day-old piglets had a relatively low platelet count ranging from 200 to 450 × 10^9/L (mean 320 × 10^9/L). For nursing piglets, the platelet counts increased significantly ($P < 0.01$) within one week to 500–630 × 10^9 counts/L and remained high thereafter (Experiments II and III). Canola oil caused a significant and consistent two-week delay in the rise of platelet counts. The transfer of piglets to solid diets at 28 d had no effect (Experiment III) and neither did increasing the level of vitamin E in the diet (Experiment I). To test whether the delay in platelet counts might be related to 22:1n-9 in canola oil, a mixture of oils was fed having a fatty acid composition similar to canola oil but devoid of 22:1n-9 (Table 1). Erucic acid at the level of ~2% did not appear to affect the delay in platelet counts, as piglets fed the mixture of oils and canola oil had a similar pattern in platelet counts (Experiments II and IV, Table 2). On the other hand, adding saturated fatty acids in the form of coconut oil to canola oil (in a 2:3 ratio) caused disappearance of the delay (Experiment III). HOAS oil also caused a two-week delay in the rise of platelet counts similar to that caused by canola oil (Experiment II).

The results with soybean oil were not consistent. In Experiments I, II and IV, soybean oil showed high platelet counts after about one week, but not in Experiment III, in which there was a one-week delay. Olive oil showed a similar inconsistency. In Experiment III, the platelet count was high after 10 d, whereas in Experiment IV the platelet count was low.

In the one-day-old piglet, the distribution of platelet size was generally biphasic, but by the second week a more bell-shaped distribution was observed. This was associated with a significant reduction in MPV in sow-reared piglets (Table 3). By the third and fourth week, sow-reared piglets had a significantly reduced MPV compared to those fed the milk replacer diets high in oleic acid (HOAS, canola and mixture of oils), but there was no significant correlation of MPV to platelet counts.

Other hematological variables. The hematological data from Experiment II are presented in Table 3. In general, the major changes in many of the parameters were related to the age of the piglets. These included a significant increase in hemoglobin (Hb), RBC counts and mean corpuscular hemoglobin concentration (MCHC),

TABLE 2

Platelet Count of Piglets Left with the Sow or Fed Milk Replacer Diets for 28 Days[a]

Experiment	Dietary groups	Days on diet 0	7	14	21	28	SEM
				($\times 10^9$/L)			
I	Soybean oil	380^{e}	$514^{b,f}$	540^{f}	$830^{b,g}$	$790^{b,g}$	56
	Canola oil[h]	322^{e}	$403^{b,c,e,f}$	420^{f}	$675^{b,c,g}$	$597^{c,g}$	31
	Canola oil[h]	381^{e}	$477^{b,e}$	415^{e}	$660^{b,c,f}$	$740^{b,f}$	44
	Canola oil[i]	302^{e}	$347^{c,e}$	470^{f}	$612^{c,g}$	$611^{c,g}$	36
	SEM	36	43	60	59	37	
	ANOVA—soy vs. canola	NS	*	NS	*	**	
	ANOVA—among canola	NS	NS	NS	NS	*	
II	Sow-reared	279^{e}	$502^{b,f}$	$614^{b,c,f}$	$926^{b,g}$	623^{f}	76
	Soybean oil	336^{e}	$530^{b,f}$	$675^{b,g}$	$719^{c,g}$	527^{f}	45
	HOAS oil	269^{e}	$291^{c,e}$	$416^{d,f}$	$567^{c,g}$	$500^{f,g}$	35
	Canola oil	339^{e}	$338^{c,e}$	$462^{c,d,f}$	$631^{c,g}$	482^{f}	50
	Mixture of oils	266^{e}	$396^{b,c,e}$	$540^{b,c,d,f}$	$682^{c,g}$	580^{f}	30
	SEM	25	55	61	67	67	
	ANOVA—all diets	NS	*	*	*	NS	
	ANOVA—among oils	*	*	*	NS	NS	
		0	10	16	28	47	
III	Sow-reared	325^{e}	$627^{b,g}$	$513^{c,d,f}$	$568^{f,g}$	$523^{c,f}$	36
	Soybean oil	382^{e}	$377^{c,e}$	$605^{b,c,f,g}$	692^{g}	$537^{c,f}$	45
	Olive oil	269^{e}	$541^{b,f}$	$623^{b,c,f,g}$	651^{g}	$594^{c,f,g}$	46
	Canola oil	313^{e}	$350^{c,e}$	414^{d-f}	$508^{f,g}$	$555^{c,g}$	63
	Canola/coconut oils (3:2)	368^{e}	$571^{b,f}$	$684^{b,g}$	684^{g}	$698^{b,g}$	43
	SEM	28	62	44	57	40	
	ANOVA—all diets	NS	**	**	NS	**	
	ANOVA—among oils	NS	**	**	NS	**	
IV	Soybean oil		483^{b}				
	Olive oil		315^{c}				
	Mixture of oils		316^{c}				
			35				
	ANOVA		***				

[a]Piglets were one day of age when placed on experiment. Values are means of six piglets/diet. SEM, pooled standard error of the mean between diets (horizontal values) and within animals (vertical values). ANOVA, analysis of variance testing for effect of diet (column) and time on diet (row); NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$. See Table 1 for other abbreviations.

[b–g]Values within an experiment and time period (column) having different superscripts *b–d*, and within a diet (row) having different superscripts *e–g*, are significantly different ($P < 0.05$).

[h]Canola oil diets contained 22 IU vitamin E per kg.

[i]Canola oil diet contained 100 IU vitamin E per kg.

or an increase in mean corpuscular volume followed by a decrease. The packed cell volume (PCV), the MPV and mean corpuscular hemoglobin (MCH) values did not change significantly throughout the four-week feeding trial, while the white blood cell (WBC) counts showed wide variation from one week to another.

In general, there were several diet differences at the one and two week measurements, but by the fourth week, all the diet differences disappeared. The values for the soybean oil-fed group were similar to sow-reared piglets, except at a few time periods in WBC, MCH and MCHC. On the other hand, compared to sow-reared piglets, the three milk replacer diets high in oleic acid (canola oil, HOAS oil and the mixture of oils) showed decreased values of PCV, Hb and RBC counts within the first two weeks, and retained higher MPV values even at four weeks on the diets.

The prothrombin time (PT) and activated partial thromboplastin time (APTT) values were high at one day of age, decreased significantly after one week, and did not change significantly thereafter (Table 3). Piglets fed soybean oil and HOAS oil showed significantly higher PT values at two and three weeks, but by the fourth week there were no significant differences between diets. The APTT values in piglets fed the milk replacer diets remained constant after the first week, while the sow-reared piglets showed some variation which, however, was not significant.

Bone marrow. The bone marrow sections of all piglets (Experiment II) were normal with the exception of three

TABLE 3

Hematological Variables of Piglets Left with the Sow or Fed Milk Replacer Diets for 28 Days (Experiment II)

Diet groups	Time on diet (d) 0	7	14	21	28	SEM	Time on diet (d) 0	7	14	21	28	SEM
	PCV (g/L)						MCV (fL)					
Sow-reared	0.37	0.40	0.39	0.40	0.37	0.02	68.9^{e}	$76.7^{a,b,f}$	$67.0^{b,e}$	$64.4^{d,e}$	58.4^{d}	1.7
Soybean oil	0.39	0.40^{a}	0.39	0.39	0.38	0.01	$70.0^{e,f}$	$72.6^{a,b,f}$	$66.6^{b,e,f}$	$65.1^{d,e}$	59.1^{d}	1.6
HOAS oil	0.34	$0.37^{b,c}$	0.37	0.38	0.38	0.01	$68.4^{d,e}$	$76.8^{a,b,f}$	$71.6^{a,b,e,f}$	$69.1^{d,e}$	62.8^{d}	1.6
Canola oil	0.39	0.35^{c}	0.37	0.38	0.38	0.01	70.0^{e}	$70.0^{b,e}$	$68.3^{a,b,d,e}$	$68.0^{d,e}$	62.1^{d}	1.6
Mixture of oils	0.38	$0.37^{b,c}$	0.39	0.41	0.37	0.01	$68.8^{d,e}$	$78.0^{a,f}$	$73.6^{a,e,f}$	69.7^{e}	62.1^{d}	1.5
SEM	0.02	0.01	0.01	0.01	0.01		1.6	2.0	1.7	1.9	1.9	
ANOVA	NS	**	NS	NS	NS		NS	*	*	NS	NS	
	Hb (g/L)						MCH (pg)					
Sow-reared	102^{d}	$109^{a,d,e}$	$113^{a,d,e}$	$113^{a,b,d,e}$	118^{e}	6	$19.3^{d,e}$	$20.5^{a,e}$	$19.7^{a,b,d,e}$	18.3^{d}	18.6^{d}	0.5
Soybean oil	107^{d}	$103^{a,d}$	$110^{a,b,d,e}$	$112^{a,b,d,e}$	121^{e}	4	19.4	$18.9^{a,b}$	18.8^{b}	18.5	18.8	0.4
HOAS oil	94^{d}	$95^{b,d,e}$	$105^{a,b,d,e}$	$109^{a,b,e,f}$	120^{f}	3	18.7	$19.9^{a,b}$	$20.1^{a,b}$	19.4	19.7	0.4
Canola oil	$109^{e,f}$	$94^{b,d}$	$101^{b,d,e}$	$106^{b,d,e}$	120^{f}	3	19.3	18.6^{b}	18.9^{b}	18.7	19.5	0.5
Mixture of oils	106^{d}	$96^{b,d}$	$110^{a,b,d,e}$	$118^{a,e}$	120^{e}	3	19.1^{d}	$20.2^{a,b,d,e}$	$20.9^{a,e}$	$20.0^{d,e}$	$19.8^{d,e}$	0.3
SEM	7	2	3	3	4		0.5	0.4	0.5	0.6	0.6	
ANOVA	NS	***	*	NS	NS		NS	*	*	NS	NS	
	RBC ($\times 10^{12}$/L)						MCHC (g/L)					
Sow-reared	5.4^{d}	$5.3^{a,b,d}$	$5.8^{a,d,e}$	6.2^{e}	6.4^{e}	0.2	280^{e}	267^{d}	$294^{a,e}$	$284^{a,b,e}$	318^{f}	4
Soybean oil	5.6^{d}	$5.5^{a,d}$	$5.8^{a,d,e}$	$6.1^{d,e}$	6.5^{e}	0.2	277^{e}	260^{d}	$283^{a,b,e}$	$284^{a,b,e}$	319^{f}	4
HOAS oil	$5.0^{d,e}$	$4.8^{b,d}$	$5.2^{b,d,e}$	$5.7^{e,f}$	6.1^{f}	0.2	273^{e}	260^{d}	$281^{b,e}$	$282^{a,b,e}$	316^{f}	4
Canola oil	$5.6^{d,e}$	$5.1^{a,b,d}$	$5.4^{a,b,d}$	$5.7^{d,e}$	6.1^{e}	0.2	276^{d}	265^{d}	$277^{b,d}$	$275^{b,d}$	316^{e}	4
Mixture of oils	$5.5^{d,e,f}$	$4.8^{b,d}$	$5.3^{a,b,d,e}$	$5.9^{e,f}$	6.1^{f}	0.2	278^{e}	259^{d}	$284^{a,b,e}$	$287^{a,e}$	321^{f}	4
SEM	0.3	0.2	0.2	0.2	0.2		3	3	3	2	6	
ANOVA	NS	**	*	NS	NS		NS	NS	**	*	NS	
	WBC ($\times 10^{9}$/L)						MPV (fL)					
Sow-reared	$11.4^{d,e}$	$13.6^{b,f}$	7.0^{d}	$6.7^{a,d}$	$9.4^{d,e}$	1.3	11.4^{f}	$9.9^{b,e}$	$9.2^{b,d,e}$	$8.9^{b,d,e}$	$8.4^{b,d}$	0.3
Soybean oil	11.1^{e}	$14.8^{b,e}$	8.1^{d}	$16.2^{b,e}$	8.7^{d}	1.9	10.9^{e}	$10.1^{a,b,d,e}$	$9.7^{a,b,d}$	$9.,8^{a,b,d}$	$9.3^{a,b,d}$	0.3
HOAS oil	9.1^{d}	$14.1^{b,e}$	7.7^{d}	$15.1^{b,e}$	8.6^{d}	1.5	10.8^{e}	$10.4^{a,b,d,e}$	$10.3^{a,d,e}$	$10.0^{a,d}$	$10.1^{a,d,e}$	0.2
Canola oil	$10.6^{d,e}$	$12.5^{b,e}$	$8.5^{d,e}$	$13.2^{b,e}$	6.5^{d}	2.0	$10.4^{d,e}$	$11.0^{a,e}$	$10.1^{a,b,d}$	$10.5^{a,d,e}$	$10.0^{a,d}$	0.2
Mixture of oils	10.5^{d}	$21.2^{a,e}$	9.3^{d}	$16.5^{b,e}$	8.7^{d}	1.3	11.0^{f}	$10.5^{a,b,e,f}$	$9.8^{a,b,d,e}$	$10.1^{a,d,e}$	$9.5^{a,b,d}$	0.3
SEM	1.6	2.0	1.9	1.8	0.9		0.12	0.13	0.13	0.12	0.17	
ANOVA	NS	*	NS	**	NS		NS	NS	NS	*	*	
	PT (s)						APTT (s)					
Sow-reared	19.3^{e}	9.8^{d}	$9.8^{b,d}$	$9.5^{c,d}$	11.7^{d}	0.7	49.3^{e}	$17.0^{b,d}$	19.0^{d}	17.9^{d}	$20.5^{a,d}$	1.0
Soybean oil	18.3^{e}	10.3^{d}	$10.7^{a,d}$	$10.8^{a,b,d}$	11.1^{d}	0.6	56.6^{e}	$19.0^{a,b,d}$	18.0^{d}	18.5^{d}	$18.0^{b,d}$	3.3
HOAS oil	18.0^{e}	9.9^{d}	$11.0^{a,d}$	$11.1^{a,d}$	11.8^{d}	1.0	46.5^{e}	$20.0^{a,d}$	18.4^{d}	18.5^{d}	$19.3^{a,b,d}$	1.6
Canola oil	17.5^{e}	10.1^{d}	$10.3^{a,b,d}$	$10.0^{b,c,d}$	11.5^{d}	0.6	50.4^{e}	$18.3^{a,b,d}$	18.4^{d}	18.7^{d}	$18.3^{b,d}$	2.9
Mixture of oils	16.9^{e}	10.1^{d}	$10.3^{a,b,d}$	$10.0^{b,c,d}$	11.5^{d}	0.8	47.5^{e}	$19.2^{a,b,d}$	19.0^{d}	18.4^{d}	$18.6^{a,b,d}$	1.7
SEM	0.9	0.4	0.2	0.2	0.3		5.2	0.7	0.5	0.3	0.5	
ANOVA	NS	NS	*	***	NS		NS	NS	NS	NS	*	

$^{a-f}$Values within a hematological variable and time period (column) having different superscripts *a, b, c,* and within a diet (row) having different superscripts *d, e, f,* are significantly diffrent ($P < 0.01$). Piglets were one day of age when placed on experiment. Values are means of six piglets/diet. SEM, pooled standard error of the mean between diets (horizontal values) and within animals (vertical values). ANOVA, analysis of variance testing for effect of diet (column) and time on diet (row); NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$. PCV, packed cell volume; Hb, hemoglobin; RBC, red blood cell; WBC, white blood cell; PT, prothrombin time; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; MPV, mean platelet volume; APTT, activated thromboplastin time.

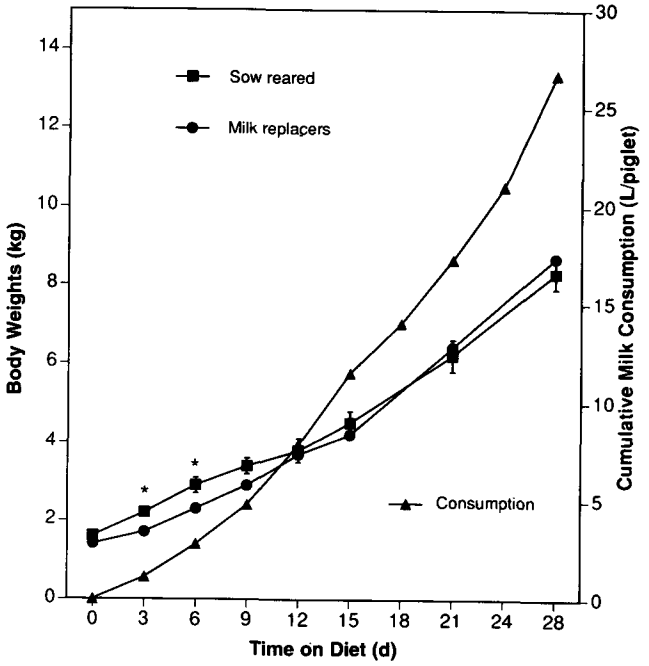

FIG. 1. Body weights of piglets left with the sow (●) or fed milk replacer diets (■) (25% by wt oil) from 1 to 28 d (Experiment II). For each time period, the pooled SEM (error bar) and significance (no label, $P > 0.05$; *$P < 0.05$) are indicated. The four milk replacer diets in Experiment II are combined because there were no significant differences between them. The average cumulative milk consumption per piglet (▲) fed milk replacer for 28 d is shown on the right side.

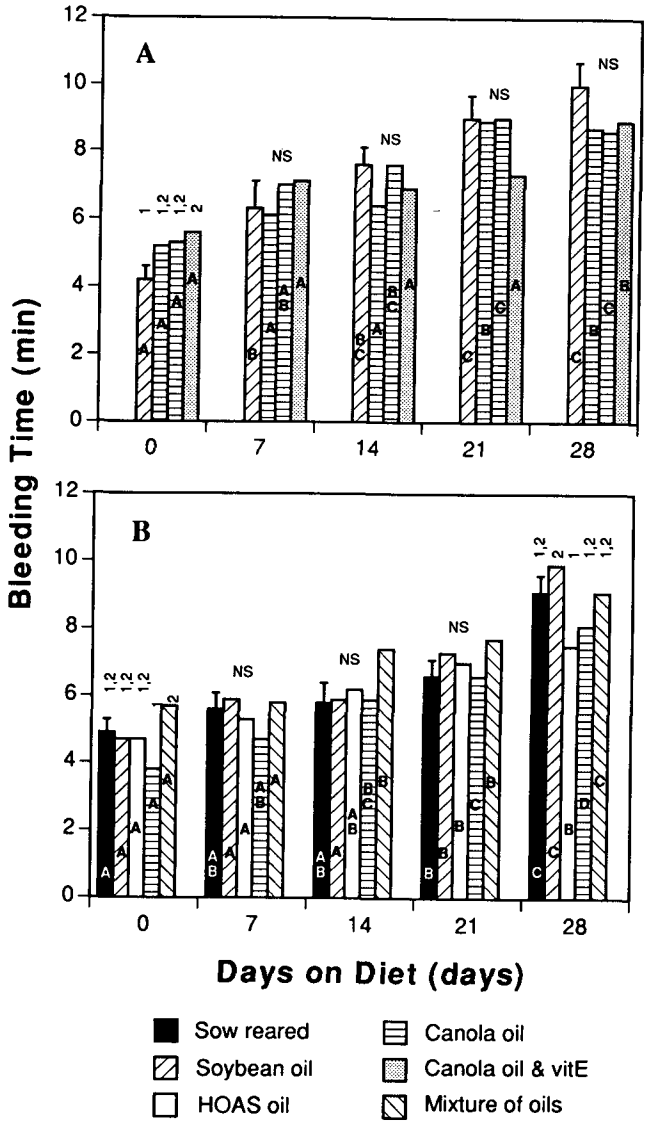

FIG. 2. Bleeding times of piglets left with the sow or fed milk replacer diets (25% by wt oil) from 1 to 28 d. For each time period and experiment, the pooled SEM is indicated as an error bar above the column. Differences between diets ($P < 0.01$) are indicated by different numbers above the columns. Differences within a diet with time are shown by different capital letters within the bars. High oleic acid sunflower (HOAS); mixture of oils, HOAS oil/soybean oil/linseed oil (55:31:14). Results of Experiments I (A) and II (B).

piglets (two fed canola oil and one fed soybean oil) which showed somewhat hypercellularity in the marrows with increased numbers of megakaryocytes at 28 d. These piglets also showed a high (>600 × 10^9/L) platelet count.

Blood lipid changes. There were characteristic differences in the plasma, RBC membrane and platelet lipids, but no differences between diets. While plasma was rich in cholesteryl esters (CE; ~36%), phosphatidylcholine (PC; ~30%), sphingomyelin (SP; ~15%) and triacylglycerol (TAG; ~11%), the RBC membranes were composed mainly of cholesterol (C; ~27%), phosphatidylethanolamine (PE; ~19%), PC (~18%), SP (~14%) and phosphatidylserine (PS; ~12%). The platelet lipid profile was generally similar to that of the RBC membrane lipids except for higher PS (~16%), TAG (~9%) and CE (~5%), and lower SP (~11%) values.

The fatty acid composition of plasma, RBC membrane and platelet PC (Table 4) and PE (Table 5) of Experiment II were selected for diet comparisons. Piglets fed soybean or canola oils, which contained appreciable amounts of 18:3n-3, showed significantly higher levels of both 18:3n-3 and its C_{22}n-3 PUFA metabolites in all three blood fractions as compared to piglets fed HOAS oil which contained little 18:3n-3. On the other hand, piglets nursing the sow received preformed C_{22}n-3 PUFA and little 18:3n-3 in their milk (Table 1), which was reflected in their relative high abundance of the n-3 PUFA and low 18:3n-3 content in the blood fractions. The concentration of 20:5n-3 in canola oil-fed piglets was significantly higher than in any of the other diets, including the nursing piglets. In plasma and RBC membrane PC and PE, the ratio of n-6 to n-3 long-chain PUFA was lowest in canola oil-fed piglets, followed by soybean oil, sow-reared, and HOAS oil-fed piglets (Tables 4 and 5). Generally, the n-6/n-3 ratio for each diet group was similar between plasma and RBC membranes within each phospholipid, and higher for PC than for PE. In platelet PC and PE, the ratio of n-6 to n-3 long-chain PUFA for all diet groups was much higher as compared to plasma and RBC membrane lipids. The n-6/n-3 PUFA ratios were

TABLE 4

Fatty Acid Composition of Phosphatidylcholine from Plasma, Red Blood Cell Membrane (RBC) and Platelets of Piglets Fed Milk Replacer Diets or Nursing the Sow for 28 Days (Experiment II)

		Saturated (Sat.) fatty acids			Monounsaturated (Mono.) fatty acids					Polyunsaturated fatty acids (PUFA)									
	Dimethyl acetals	16:0	18:0	Σ Sat.	18:1n-9	18:1n-7	20:1n-9	22:1n-9	Σ Mono.	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:4n-6	22:5n-6	22:5n-3	22:6n-3	Σ C_{22} PUFA	≥ C_{20} n-6/n-3
										(area %)									
Plasma																			
Sow milk	0.1[a]	21.5	28.3	43.5	12.9	2.2	0.1	0	17.1	20.9	0.2	9.7	0.1	0.5	0.4	0.7	1.3	2.9	4.7
Soybean oil	0.1	16.5	28.7	45.6	9.2	1.7	0.2	0	11.3	30.8	0.9	7.0	0.2	0.1	0	0.8	1.3	2.2	3.2
HOAS oil	0.1	12.1	28.1	40.6	29.7	1.2	0.4	0	32.1	15.6	0.1	8.2	trace[b]	0.4	0.6	0.2	0.5	1.7	11.6
Canola oil	0.1	13.1	25.0	38.9	23.5	4.2	1.2	0.1	29.8	20.7	1.4	4.7	0.6	0.1	0	0.6	1.3	2.0	2.1
SEM[c]	0.07	1.6	1.4	2.8	0.9	0.2	0.04	0	1.0	1.0	0.08	1.0	0.08	0.05	0.06	0.12	0.2	0.5	0.4
ANOVA[d]	NS	*	NS	NS	***	***	***	***	***	***	***	*	**	**	**	**	**	NS	***
RBC membrane																			
Sow milk	0.2	41.4	11.8	53.9	19.1	2.2	0.1	trace	23.4	16.5	0.2	3.8	0.1	0.1	0.1	0.2	0.4	0.9	6.0
Soybean oil	0.2	30.7	17.6	49.1	15.3	1.8	0.1	0	17.9	27.6	0.8	2.6	0.1	0	0	0.2	0.4	0.7	4.1
HOAS oil	0.2	27.3	13.5	41.5	37.6	1.4	0.3	0	40.4	13.5	0.1	3.1	0.1	0.1	0.2	0.1	0.2	0.5	11.0
Canola oil	0.2	29.5	14.3	44.6	29.7	3.5	0.5	0.1	34.7	16.2	1.0	1.5	0.2	0	0	0.1	0.3	0.5	2.8
SEM	0.06	2.1	0.9	2.8	0.7	0.06	0.02	0.02	0.6	1.8	0.1	0.5	0.04	0.02	0.02	0.04	0.1	0.2	0.9
ANOVA	NS	**	*	*	***	***	***	**	***	**	***	NS	NS	*	**	*	*	NS	**
Platelet																			
Sow milk	0.2	54.1	12.0	66.9	17.6	2.7	0.2	0	22.6	6.1	trace	2.7	0.1	0.1	0	0	0	0.1	17
Soybean oil	0.2	40.1	19.5	60.5	17.3	2.3	0.3	0.1	22.0	12.5	0.3	2.6	0.1	0.1	0	0.1	0.2	0.4	9
HOAS oil	0.2	36.6	13.3	50.9	33.3	1.6	0.7	0.1	37.1	6.0	0	4.5	0.1	0.1	0	0	0	0.1	26
Canola oil	0.2	32.3	12.8	46.2	29.3	4.1	1.2	0.3	36.7	10.7	0.6	3.3	0.5	0.1	0	0.1	0.2	0.3	5

[a]All values are means of three piglets/diet. [b]Trace (<0.05%). [c]SEM, pooled standard error of the mean; HOAS, high oleic acid sunflower. [d]ANOVA, analyses of variances testing for diet NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 5

Fatty Acid Composition of Phosphatidylethanolamine in Plasma, Red Blood Cell Membrane (RBC) and Platelets of Piglets Fed Milk Replacer Diets or Nursing the Sow for 28 Days (Experiment II)

		Saturated fatty acids			Monounsaturated fatty acids					Polyunsaturated fatty acids (PUFA)									
	Dimethyl acetals	16:0	18:0	Σ Sat.	18:1n-9	18:1n-7	20:1n-9	22:1n-9	Σ Mono.	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:4n-6	22:5n-6	22:5n-3	22:6n-3	Σ C_{22} PUFA	≥ C_{20} n-6/n-3
										(area %)[e]									
Plasma																			
Sow milk	4.5[a]	13.4	21.6	37.0	16.5	1.9	0.3	0.1	19.9	16.9	0.6	9.3	0.2	3.3	1.1	2.9	4.2	11.5	2.2
Soybean oil	3.3	14.7	33.2	51.4	15.4	1.1	0.1	trace[b]	22.3	16.1	1.0	4.8	0.1	0.4	0.1	0.4	1.0	2.0	4.1
HOAS oil	6.1	13.5	26.3	47.3	25.7	0.3	0.4	0.1	33.3	9.8	0.8	5.0	0.1	0.9	0.7	0.2	0.8	2.6	6.3
Canola oil	2.7	12.3	20.0	36.2	25.9	3.5	1.1	0.7	34.9	16.0	2.0	4.9	0.4	0.4	0.1	1.1	2.5	4.1	1.5
SEM[c]	2.3	2.2	3.5	6.1	2.8	0.3	0.1	0.2	3.7	1.8	0.3	0.9	0.1	0.3	0.1	0.4	0.6	1.6	0.6
ANOVA[d]	NS	NS	NS	NS	*	***	**	**	*	NS	*	*	NS	**	**	**	**	**	***
RBC membrane																			
Sow milk	2.7	27.4	6.9	34.7	33.0	2.1	0.1	0	38.0	16.3	0.3	4.9	0.1	1.2	0.5	1.4	1.5	4.8	2.4
Soybean oil	1.1	27.1	9.3	36.7	27.7	1.9	0.1	0	30.6	25.8	1.2	1.8	0.1	0.4	0.1	0.8	1.1	2.4	1.5
HOAS oil	1.0	18.3	6.7	25.3	55.2	1.7	0.2	0	57.9	11.5	0.1	2.5	0.1	0.6	0.4	0.2	0.4	1.5	6.1
Canola oil	1.9	21.0	7.5	28.9	45.7	2.8	0.1	0.1	50.0	13.8	1.6	1.8	0.2	0.2	0.1	0.9	1.2	2.4	1.2
SEM	0.3	2.4	0.7	2.9	1.8	0.1	0.08	0.02	1.7	1.0	0.3	0.5	0.03	0.1	0.1	0.2	0.3	0.8	0.2
ANOVA	*	*	NS	*	***	***	NS	*	***	***	***	**	NS	**	*	**	**	*	***
Platelet																			
Sow milk	25.2	17.2	19.0	37.5	21.4	2.8	0.2	0	28.8	5.1	0.6	21.2	0.3	2.7	0.4	0.6	0.4	4.1	16
Soybean oil	9.3	10.5	20.9	32.2	20.0	2.8	0.2	0.1	24.6	13.5	0.6	21.1	0.7	2.6	0.1	1.0	0.7	4.4	10
HOAS oil	12.4	13.9	18.9	33.2	32.2	1.8	0.6	0.1	36.6	3.4	0	20.3	0.1	3.0	0.5	0.2	0.2	3.9	41
Canola oil	9.4	16.1	20.4	37.3	29.2	4.0	0.8	0.2	36.5	7.6	0.7	11.7	1.0	1.5	0	0.9	0.3	2.7	6

[a–d]For footnotes *a* to *d* see Table 4. [e]All dimethylacetal values are percent of total dimethylacetal and fatty acid methyl ester responses, while the fatty acid data are expressed as percent of total fatty acid methyl esters only. Other abbreviations as in Table 4.

highly correlated (0.97–0.99) to the dietary n-6/n-3 ratios (calculations not shown).

The relative concentration of 18:2n-6 in the PC and PE was proportional to that present in the diet (calculations not shown). The concentration of arachidonic acid (20:4n-6) in plasma and RBC membranes, but not in platelets, was significantly higher in sow-reared piglets compared to those fed the milk replacer diets because this acid was present in sow milk. Among the milk replacer diets, there were few differences in 20:4n-6; generally, canola oil was on the low side and HOAS on the high side.

The monounsaturated fatty acids generally paralleled those found in the diet, and the incorporation of 22:1n-9 was minimal (Tables 4 and 5). The relative concentration of the saturated fatty acids was generally lower in the canola and HOAS oil-fed piglets compared to the soybean oil-fed and sow-reared piglets. The alkenyl ether content, measured as dimethylacetals, was greatest in platelet PE; on the milk replacer diets their levels were reduced significantly.

DISCUSSION

In this study, newborn piglets were fed milk replacers containing soybean or canola oils at 25% by weight of dry matter content providing each piglet with 1.5 g/d 18:3n-3 at 3 d, 4.9 g/d at 15 d and 5.8 g/d at 28 d of age, or approximately 1 g/d/kg body weight (calculated from results in Fig. 1). This consumption of 18:3n-3 from canola or soybean oils by the newborn piglet is approximately ten times greater per kg body weight than is provided in adult human trials (in Ref. 29, canola oil was fed at 7.5 g/d or 0.1 g/d/kg body weight for a 75 kg person). Based on the increased consumption relative to body weights of a newborn compared to an adult, there is certainly a need to test the newborn using dietary oils containing 18:3n-3. Furthermore, the newborn is undergoing a rapid transition from oxidizing glucose (in the fetal state) to oxidizing fatty acids as the main source of energy (43), and may therefore be more sensitive to dietary changes.

The results of these studies show that the bleeding times increased with age over the first four weeks on all diets. Piglets fed about 1 g/d/kg 18:3n-3 from canola or soybean oil diets, or those nursing the sow, which received preformed long-chain n-3 PUFA, showed prolonged bleeding times after four weeks compared to HOAS oil feeding (Fig. 2). The increased bleeding times in the canola and soybean oil groups would appear to be due to the piglet's ability to convert dietary 18:3n-3 into 20:5n-3 and 22:6n-3, as evidenced by the higher levels of these n-3 PUFA in blood lipids of these piglets compared to those fed HOAS oil (Tables 4 and 5). The increase in long-chain n-3 PUFA in blood lipids in response to dietary 18:3n-3 has been observed by other investigators (20,22,24,27), but because all but one group (24,29) did not measure bleeding times concurrently, the possible risk could not be assessed. Furthermore, this is the first study in which bleeding has been tested in the newborn.

Three studies with adult humans were reported recently in which diets were formulated to contain substantial amounts of canola oil (about 10% 18:3n-3) that provided subjects with 3 (25) and 7.4 g/d (27,29) 18:3n-3, or 0.04 and 0.1 g/d/kg body weight, respectively. In two studies a significant increase in bleeding time was observed, namely from 4.3 to 6.7 min (25) and 3.9 to 4.9 min (29). The latter group (29) did not confirm increased bleeding times in their repeat study (27). However, it should be noted that all these bleeding times are within the normal range accepted for humans (11,44). One would conclude from these results that consumption of vegetable oils with about 10% 18:3n-3 as sole source of fat in the diet appears to provide a low risk of excessive bleeding in adults (25,27,29). A similar conclusion can be made from the results of the present study of feeding canola oil or soybean oil as sole source of fat to newborn piglets. The bleeding times increased significantly ($P < 0.001$) from about 4 to 6 min to 7 to 10 min, but the risk for bleeding would appear to be low, except possibly for hemophiliacs.

Piglets fed milk replacer diets often showed a transient delay in the normal rise of platelet counts within the first week of life compared to sow-reared piglets. This delay in the rise of platelet counts was consistent and more prolonged for canola and HOAS oil-fed piglets compared to soybean oil and olive oil-fed piglets. It would appear that this phenomenon was related to the fatty acid composition of the oil in the milk replacer, as the delay was reproduced by mimicking the fatty acid profile of canola oil, and eliminated by the addition of saturated fatty acids in the form of coconut oil to canola oil. A low level of dietary 16:0 was recently shown to be one of the factors associated with lower platelet counts in piglets even in the presence of high levels of shorter chain saturated fatty acids (20%) in the diet (28). If the effects of saturated fatty acids on platelet number can be verified, it would alleviate the concern of reduced platelet counts in the newborn fed diets containing vegetable oils, all of which are generally low in saturated fatty acids. It is of interest to note that *de novo* synthesis of fatty acids in piglets during the first three weeks after birth is negligible (45,46), which coincides with the time of reduced platelet counts. A low availability of saturated fatty acids, both from the diet and *de novo* synthesis, would lead to lower levels of total saturated fatty acids in membrane lipids, which was in fact observed in all three blood components from canola and HOAS oil-fed piglets (Tables 4 and 5). As cell membranes contain approximately 40% saturated fatty acids (47), a reduction in this level could lead to altered function and/or physical characteristics.

Diets high in 22:1n-9 have been associated with reduced platelet counts in humans (48–50). The results of the present study show that the delay in the rise of platelet counts is associated with fatty acids other than 22:1n-9 as a similar pattern was observed with oils and oil mixtures devoid of 22:1n-9 as with canola oil. The question of whether 22:1n-9 at levels in excess of that permitted in canola oil, i.e., 2% (33), will cause a further reduction in platelet counts in the newborn, remains to be investigated.

There was no correlation between increased bleeding

and decreased platelet counts in this study, as was observed by others (5,10,50). Furthermore, we observed no increase in PT and APTT in response to increased bleeding in agreement with studies with fish oil (5,7,14,15,16) and 22:1n-9 (50). A number of hematological parameters showed temporary changes (PCV, Hb and RBC counts) which appeared to be related to diets high in 18:1n-9, but these differences disappeared by the fourth week on the diet. Most hematological values were within the normal range reported for piglets (32,51).

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The Lipids of Slugs and Snails: Evolution, Diet and Biosynthesis

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There is a considerable gap in current knowledge of the lipid composition of snails and slugs, both of which belong to the phylum Mollusca. We have therefore analyzed the sterol and fatty acid compositions of three species of slugs and three species of snails. The sterols of slugs included eight different sterols: cholesterol contributed 76-85% of the total sterols, brassicasterol accounted for 4-13%; other sterols we identified were lathosterol, 24-methylene cholesterol, campesterol, stigmasterol, sitosterol and sitostanol. In contrast, snails contained two additional sterols, desmosterol and cholestanol. Of the polyunsaturated fatty acids in slugs, linoleic (18:2n-6) and arachidonic acids (20:4n-6) were the major n-6 fatty acids, while linolenic (18:3n-3) and eicosapentaenoic acids (20:5n-3) were the predominant n-3 fatty acids. Docosahexaenoic acid (22:6n-3), the end product in the n-3 fatty acid synthetic pathway and an important membrane fatty acid of mammals, fish and birds, was absent in both slugs and snails. However, the analogous product of n-6 fatty acid synthesis, 22:5n-6, was found in both snails and slugs. This raises speculation about preference for n-6 fatty acid synthesis in these species. Our data show the unique sterol and fatty acid compositions of slugs and snails, as well as similarities and differences in sterol composition between the two. The results between the two land mollusks are contrasted with those of marine mollusks, such as oysters, clams and scallops.

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Mollusks as a group have a unique sterol and fatty acid composition. Previously, we reported that oysters, clams and scallops contain many other sterols besides cholesterol, including 24-methylene cholesterol and brassicasterol (1,2). These shellfish sterols are variably absorbed by the human intestine (1). Shellfish also have a high concentration of the biologically important longer chain n-3 fatty acids (3). However, detailed information about the sterol and fatty acid composition of slugs and snails is lacking, especially for slugs (3–14). Both are members of the same phylum, Mollusca, with slugs having evolved from snails (15).

In the present study, we have analyzed the sterol and fatty acid composition of three species of slugs and three species of snails. These land mollusks had quite different fatty acids and sterols than the mollusks of the sea such as clams, oysters and scallops. The nutrition of the marine mollusks is derived from phytoplankton, the plants of the sea, and zooplankton, whereas the diet of slugs and snails consists of land-based vegetation. In both species, cholesterol synthetic precursors were found, desmosterol in snails and lathosterol in slugs, and in both we observed the complete absence of the n-3 docosahexaenoic acid.

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Abbreviation: TLC, thin-layer chromatography.

MATERIALS AND METHODS

Three species of slugs were collected from local gardens in Portland, Oregon, between May and June of 1992–1993, and were classified according to "Plants and Animals of the Pacific Northwest" (15). They included *Prophysaon andersoni*, *Arion ater* (coal-black phase and reddish-brown phase) and *Limax maximus*. *Prophysaon andersoni* was 5–6 cm long and weighed 0.2–0.5 g, with lemon-yellow color. In the coal-black phase, *A. ater* was 5–7 cm, 0.8–2.1 g. In the reddish-brown phase, *A. ater* had the same length as in the coal-black phase, but was heavier (1.2–2.8 g). *Limax maximus*, a gray color, was the longest and heaviest among the different species of slugs studied (length, 8–11 cm; weight, 2.3–7.8 g). We also analyzed three different species of snails: one species of canned snail (*Helix* sp., from France) and two species of live snails collected in the local gardens (*Vespericola columbiana and Hapotrema sportella*). They differed in weight and color of the shell. *Helix* sp., the heaviest among them (7.2–10.0 g without the shell), had a light-brown shell with dark-brown strips. *Vespericola columbiana* was the lightest (0.4–0.6 g) with a nearly black shell. *Haplotrema sportella* weighed 2.0–2.9 g, with a brown shell color.

The slugs and snails were killed by exposure to diethyl ether vapor. The entera and slime were removed. The tissues were freeze-dried and then ground. The lipids were extracted by a modified method of Folch *et al.* (16). The sterol composition was determined by saponification, extraction, digitonin precipitation and gas–liquid chromatography, as has been described (17). Briefly, slug lipids were saponified with alcoholic KOH. Free sterols were precipitated with digitonin (18). Digitonin precipitable sterols were then converted to trimethylsilyl ethers and subjected to gas–liquid chromatography using cholestane as an internal standard. The analyses were performed by gas–liquid chromatography on an instrument equipped with a hydrogen flame-ionization detector (model Sigma 3B; Perkin-Elmer, Norwalk, CT) and a 30-m SE-30 fused silica capillary column. Temperatures of column, detector and injection port were 260, 300 and 300°C, respectively. Helium was used as the carrier gas. [4-^{14}C]Cholesterol (New England Nuclear, Boston, MA) was used as internal standard to monitor any losses. The recovery varied between 80–90%.

The fatty acid compositions of slug and snail lipids were analyzed by methods described previously (19). Aliquots of lipid extracts were saponified with alcoholic KOH. Neutral lipids were removed by hexane extraction. Fatty acids were recovered by acidifying the aque-

ous phase, and extracting with hexane. Free fatty acids were then methylated with boron trifluoride in methanol (20). To analyze the fatty acid compositions of phospholipids, another aliquot of lipid extract was subjected to thin-layer chromatography (TLC) using the solvent system hexane/chloroform/diethyl ether/acetic acid (80:10:10:1, by vol) (21), and lipid classes (phospholipids, free fatty acid, triglycerides and cholesteryl esters) were separated. The fatty acids of phospholipids were transmethylated with boron trifluoride in methanol as described. Methyl ester of fatty acids were analyzed by gas–liquid chromatography on an instrument equipped with a hydrogen flame-ionization detector (Perkin-Elmer model 8500) and a 30-m SP-2330 fused silica capillary column. Temperatures of column, detector and injection port were 184, 250 and 250°C, respectfully. Helium was used as the carrier gas. The retention time and area of each peak were measured on a dedicated chromatography station running Omega (Perkin-Elmer). A mixture of fatty acid standards was run daily for calibration purposes.

RESULTS

Sterol composition. The sterol compositions of the three species of slugs are given in Table 1. Eight different sterols were found which contained 63 to 104 mg sterols per 100 g wet tissue. Cholesterol was the major sterol (76–85% of total sterols). Brassicasterol was the next most common sterol (4–13%). Three to five percent of the slug sterols were plant sterols in which campesterol, a 28 carbon sterol, predominated. Plant sterols included campesterol, stigmasterol and sitosterol, which are the three most abundant sterols in higher plants (22). Other sterols, e.g., brassicasterol, are found in a few land plants (23). The sterol compositions were similar between different species of slugs although small differences did exist. For example, *P. andersoni* had a lower cholesterol content than the other two species of slugs.

The sterol compositions of the three species of snails are shown in Table 2. Although the sterols were similar among the different species, snails differed from slugs in containing cholestanol (1.4 to 4.1 mg/100 g wet tissue) and desmosterol (0.5 to 9.1 mg/100 g wet tissue), both of which were absent in slugs. 24-Methylene cholesterol was more consistently present in the snails. On the other hand, slugs had a higher brassicasterol content than snails. Another sterol in the pathway of cholesterol biosynthesis, 7-dehydrocholesterol, was not found.

In Table 3, the sterol compositions of slugs are compared to those of other mollusks from the sea. Although the total sterol contents were similar, the sterol compositions of slugs and snails were vastly different from those of marine shellfish. In slugs and snails, nearly 90% of the sterols were cholesterol. The other sterols included brassicasterol, campesterol, sitosterol, cholestanol, desmosterol and small amounts of lathosterol and 24-methylene cholesterol. In contrast, the cholesterol only amounted to about 40% of total sterols in filter-feeding mollusks such as scallops, oysters and clams. The other sterols were a C_{26} sterol, 22-dehydrocholesterol, brassicasterol, 24-methylene cholesterol and a C_{29} sterol. Only cholesterol was present in crab, shrimp and lobster.

Fatty acid composition. When the lipid classes (phospholipids, free fatty acids, triglycerides and steryl esters) of slugs and snails were fractionated, only the phospholipid and sterol bands were clearly seen on the TLC plate. The phospholipid fraction contained the same fatty acid compositions as did the total lipid fraction (data not shown). This suggested that the phospholipids constituted the predominate lipids in slugs and snails.

The fatty acid compositions of slugs and snails were similar among different species (Tables 4 and 5). Slug lipids contained a particularly high level of polyunsaturated fatty acids (51–57%), but docosahexaenoic acid, offer an important membrane n-3 fatty acid, was not detected in slugs although its immediate precursor, 22:5n-3, was present. Slugs contained 19–24% of saturated and 17–20% of monounsaturated fatty acids. Palmitic and stearic acids were the major saturated

TABLE 1

The Sterol Content and Composition of Different Species of Slugs

Sterol (mg/100 g wet wt)	*Prophysaon andersoni* (n = 18)	*Arion ater* (n = 18)	*Limax maximus* (n = 6)
		(means ± SD)	
Total sterols	63.3 ± 19.5[a] (100)[d]	103.6 ± 38.2[b] (100)	101.1 ± 19.5[b] (100)
Cholesterol	52.6 ± 16.7[a] (83)	91.6 ± 3.6[b] (88)	93.1 ± 3.3[b] (92)
Brassicasterol	8.0 ± 2.6[a] (13)	5.9 ± 2.9[b] (6)	4.3 ± 1.4[c] (4)
Lathosterol	0.1 ± 0.1[a] (<0.5)	0.3 ± 0.1[b] (<0.5)	0.4 ± 0.2[b] (<0.5)
24-Methylene cholesterol	ND[e]	0.1 ± 0.1[b] (<0.5)	0.5 ± 0.4[a] (1)
Total plant sterols	2.7 ± 0.8[a] (4)	5.5 ± 1.4[b] (5)	2.7 ± 0.3[a] (3)
Campesterol	1.9 ± 0.7[a] (3)	2.7 ± 1.1[b] (3)	1.7 ± 0.3[a] (2)
Stigmasterol	0.3 ± 0.3[a] (1)	1.1 ± 0.6[b] (1)	ND (< 0.5)
Sitosterol	0.5 ± 0.8[a] (1)	1.4 ± 1.5[a] (1)	0.9 ± 0.4[a] (1)
Sitostanol	ND	0.3 ± 0.5[b] (<0.5)	0.1 ± 0.1[b] (<0.5)

[a,b,c]Values with unlike superscripts are different at $P < 0.05$.
[d]Values in parentheses represent percent of total sterols.
[e]Not detected (<0.3 mg/100 g wet wt).

TABLE 2

The Sterol Content and Composition of Different Species of Snails

Sterol (mg/100 g wet wt)	*Helix* sp. (n = 5)		*Haplotrema sportella* (n = 3)		*Vespericola columbiana* (n = 2)	
	(means ± SD)					
Total sterols	99.3 ± 48.8	(100)[c]	130.1 ± 18.5	(100)	134.9 ± 29.2	(100)
Cholesterol	86.6 ± 41.5	(86)	118.4 ± 14.0	(91)	116.0 ± 21.4	(86)
Cholestanol	4.1 ± 2.5	(5)	1.8 ± 0.5	(1)	1.4 ± 1.0	(1)
Brassicasterol	1.0 ± 1.5	(1)	2.9 ± 0.3	(2)	1.1 ± 1.6	(1)
Desmosterol	0.5 ± 0.6[a]	(1)	2.7 ± 1.9	(2)	9.1 ± 5.7[b]	(6)
Lathosterol	0.1 ± 0.1	(<0.5)	0.2 ± 0.1	(<0.5)		
24-Methylene cholesterol	0.1 ± 0.0[a]	(<0.5)	0.3 ± 0.0[b]	(<0.5)	1.0 ± 0.5	(1)
Total plant sterols	6.9 ± 3.7	(7)	3.8 ± 1.7	(3)	6.3 ± 0.9	(5)
Campesterol	2.8 ± 2.0	(3)	1.7 ± 1.0	(1)	3.2 ± 0.4	(2)
Stigmasterol	0.1 ± 0.0	(<0.5)	0.1 ± 0.0	(<0.5)	0.5 ± 0.3	(<0.5)
Sitosterol	3.4 ± 1.7	(4)	2.0 ± 0.6	(2)	2.8 ± 0.8	(2)
Sitostanol	0.5 ± 0.4	(1)	0.1 ± 0.0	(<0.5)	0.1 ± 0.0	(<0.5)

[a,b]Values with unlike superscripts are different at $P < 0.05$.
[c]Percentage of total sterol in parentheses.

fatty acids. Oleic acid was the most prevalent monounsaturated fatty acid. Seventy-seven to eighty-one percent of the polyunsaturated fatty acids were of the n-6 family and 19–23% were of the n-3 family. The arachidonic acid content was especially high (13.5–14.4%) and was higher than the content of linoleic acid (11.6–15.2%). Linolenic and eicosapentaenoic acids were the predominant n-3 fatty acids.

The fatty acid compositions of slug and snail lipids showed small but significant differences. Slugs had a higher concentration of n-3 fatty acids and a lower n-6 fatty acid concentration than snails. The major n-3 and n-6 fatty acids which contributed to this difference were linolenic acid (18:3n-3) and arachidonic acid (20:4n-6). As a result, the slug lipids had a lower n-6/n-3 ratio than snail lipids. Of interest was that both species had n-6 fatty acids with a chain length of 22 carbons, i.e., 22:4n-6 and 22:5n-6.

The fatty acid compositions of slugs and snails are compared with marine mollusks and other shellfish in Table 6 and Figures 1 and 2. The most striking difference was the presence of docosahexaenoic acid in all of the shellfish from 8–27% of total fatty acids. In contrast, slugs and snails contained no docosahexaenoic acid. Slugs and snails had 3–4% eicosapentaenoic acid (20:5n-3) which was 20–30% of total fatty acids in shellfish . Both slugs and snails had a much higher content of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) than shellfish (about 4–7 times higher). Overall, slugs and snails had higher levels of n-6 fatty acids and lower levels of n-3 fatty acids than shellfish (Figs. 1 and 2) as might be expected from land based animals (n-6 predominant) compared to sea animals with predominant n-3 fatty acids.

DISCUSSION

In the present study, we have, for the first time, fully characterized the sterol and fatty acid compositions of slugs and added information about the lipids of snails. Several unique features of their lipid composition were observed. The absence of docosahexaenoic acid and the presence of other highly polyunsaturated, long-chain fatty acids in the membrane phospholipids were remarkable. A second finding was the presence of cholesterol biosynthetic precursors, desmosterol and lathosterol.

Docosahexaenoic acid is an important component of membrane phospholipids of most animals, birds and

TABLE 3

The Comparison of the Content of Major Sterols of Slugs, Snails and Marine Shellfish[a]

	Cholesterol	C_{26} Sterol	22-Dehydrocholesterol	Brassicasterol	24-Methylene cholesterol	C_{29} Sterol	Other sterols[b]	Total sterols
Slugs[c]	86.6			5.9	0.1		10.4	102.8
Snails[c]	102.0			1.6	0.1		11.9	115.6
Clams	49.0	4.4	16.0	19.0	24.3	5.0		117.6
Oysters	59.6	6.2	12.0	42.6	35.6	5.0		160.8
Scallops	51.8	13.9	25.6	30.4	25.6	17.4		164.6
Crabs	99.4							99.3
Lobsters	82.7							82.7
Shrimp	160.3							160.3

[a]Information on different marine shellfish is from references 1 and 2.
[b]Slugs and snails have other sterols, unlike shellfish, i.e., lathosterol, campesterol, etc. (see Tables 1 and 2).
[c]Values are means for raw weight after evisceration.

TABLE 4

The Fatty Acid Composition of Different Species of Slugs

Fatty acids	*Prophysaon andersoni* (n = 18)	*Arion ater* (n = 18)	*Limax maximus* (n = 6)
	(percent of total fatty acids, mean ± SD)		
Saturated			
16:0	8.0 ± 1.4[a]	6.7 ± 1.3[b]	10.1 ± 0.8[c]
18:0	9.1 ± 0.4[a]	9.1 ± 0.4[a]	10.7 ± 0.9[b]
Total saturated	20.7 ± 1.9[a]	18.9 ± 1.5[b]	23.6 ± 1.0[c]
Monounsaturated			
16:1n-7	1.3 ± 0.3[a]	1.1 ± 0.4[a]	1.1 ± 0.3[a]
18:1n-9/n-7	12.1 ± 0.6[a]	14.5 ± 1.2[b]	15.7 ± 1.3[c]
20:1n-9	3.9 ± 1.0[a]	3.3 ± 2.5[a]	3.4 ± 1.7[a]
Total monounsaturated	17.2 ± 0.6[a]	18.8 ± 2.7[b]	20.2 ± 1.3[b]
n-6			
18:2n-6	12.7 ± 0.4[a]	15.2 ± 1.6[b]	11.6 ± 0.9[c]
20:2n-6	8.9 ± 0.8[a]	9.9 ± 0.9[b]	7.9 ± 0.6[c]
20:4n-6	14.4 ± 0.5[a]	14.3 ± 1.0[a,b]	13.5 ± 1.0[b]
22:4n-6	3.8 ± 0.8[a]	4.4 ± 0.6[b]	4.3 ± 0.6[a,b]
22:5n-6	1.0 ± 0.1[a]	1.0 ± 0.2[a]	0.9 ± 9.1[b]
Total n-6	43.5 ± 2.3[a]	46.0 ± 2.0[b]	39.8 ± 1.5[c]
n-3			
18:3n-3	6.0 ± 0.7[a]	5.3 ± 1.3[b]	1.5 ± 0.3[c]
20:5n-3	4.6 ± 0.5[a]	3.7 ± 1.2[b]	7.0 ± 0.8[c]
22:5n-3	1.8 ± 0.1[a]	1.6 ± 0.2[b]	2.1 ± 0.4[c]
22:6n-3	ND[d]	ND	ND
Total n-3	13.1 ± 0.7[a]	10.8 ± 1.0[b]	11.4 ± 1.4[b]
Total polyunsaturated	56.7 ± 2.3[a]	56.9 ± 2.2[b]	51.2 ± 1.4[c]
n-6/n-3	3.3 ± 0.2[a]	4.3 ± 0.5[b]	3.6 ± 0.6[a]

[a,b,c]Values with unlike superscripts are different at $P < 0.05$.
[d]Not detected (<0.005%).

TABLE 5

The Fatty Acid Composition of Different Species of Snails

Fatty acids	*Helix* sp. (n = 5)	*Haplotrema sportella* (n = 3)	*Vespericola columbiana* (n = 2)
	(percent of total fatty acids, mean ± SD)		
Saturated			
16:0	7.1 ± 1.0[a]	9.6 ± 0.1[b]	8.0 ± 0.4
18:0	10.4 ± 0.9	10.6 ± 1.1	8.9 ± 0.2
Total saturated	19.9 ± 2.0	22.3 ± 1.4[b]	17.5 ± 1.1[a]
Monounsaturated			
16:1n-7	1.4 ± 0.4[a]	1.5 ± 0.2	3.2 ± 1.1[b]
18:1n-9/n-7	14.2 ± 1.1[a]	11.9 ± 1.5[b]	10.0 ± 0.1[b]
20:1n-9	2.2 ± 0.2[a]	0.9 ± 0.1[b]	3.6 ± 0.8[a]
Total monounsaturated	18.0 ± 0.8[a]	14.8 ± 1.8[b]	16.8 ± 0.2
n-6			
18:2n-6	15.7 ± 0.4[a]	14.5 ± 0.3[b]	11.0 ± 1.6[c]
20:2n-6	12.1 ± 0.5[a]	10.5 ± 0.5[b]	9.1 ± 2.0[b]
20:4n-6	14.8 ± 2.2	13.8 ± 0.4[a]	16.9 ± 1.6[b]
22:4n-6	4.8 ± 0.7	5.3 ± 0.2[a]	4.0 ± 0.4[b]
22:5n-6	0.3 ± 0.2	0.4 ± 0.0[a]	0.7 ± 0.1[b]
Total n-6	50.3 ± 2.7	48.8 ± 0.4	45.9 ± 2.3
n-3			
18:3n-3	1.8 ± 0.6[a]	3.2 ± 1.2	4.3 ± 1.0[b]
20:5n-3	2.8 ± 0.1[a]	1.5 ± 0.3[b]	5.5 ± 0.9[c]
22:5n-3	1.3 ± 0.4	1.7 ± 0.4	2.0 ± 1.0
22:6n-3	ND[d]	ND	ND
Total n-3	6.6 ± 0.7[a]	7.2 ± 0.6[a]	11.9 ± 2.7[b]
Total polyunsaturated	57.2 ± 2.3	56.4 ± 0.6	58.3 ± 4.3
n-6/n-3	7.8 ± 1.3	6.9 ± 0.6	5.2 ± 2.4

[a,b,c]Values with unlike superscripts are different at $P < 0.05$.
[d]Not detected (<0.005%).

TABLE 6

Comparison of Fatty Acid Compositions of the Slugs, Snails and Marine Shellfish

Fatty acid	Slug	Snail	Shellfish[a] Oyster	Clam	Scallop	Shrimp	Lobster	Crab
	(mean percent of total fatty acids)							
Saturated								
16:0	7.3	8.0	18.9	19.4	21.5	21.5	14.4	13.1
18:0	9.4	10.2	3.4	8.3	7.1	3.3	8.9	5.9
Total saturated	20.0	20.1	26.8	29.5	33.9	25.8	24.8	19.3
Monounsaturated								
16:1n-7	1.1	1.8	3.4	4.1	3.6	6.2	5.4	4.9
18:1n-9/n-7	14.3	12.6	9.4	6.7	7.3	22.7	13.2	16.6
20:1n-9	3.4	2.1	2.9	11.9	0.6	2.2		2.0
Total monounsaturated	19.0	16.8	17.2	22.7	14.0	31.1	18.6	23.5
n-6								
18:2n-6	14.4	14.4	1.6	2.2	0.8	1.2	2.0	0.7
20:2n-6	9.4	11.0	0.1					
20:4n-6	14.1	14.9	1.4	1.9	1.6	2.7	19.8	4.1
22:4n-6	4.3	4.8	1.5					
22:5n-6	1.0	0.4	0.6					
Total n-6	44.7	49.0	5.6	4.1	2.8	4.2	22.8	5.2
n-3								
18:3n-3	4.9	2.7	2.2			2.4		
20:5n-3	4.2	3.0	20.6	11.0	23.5	21.1	14.1	30.2
22:5n-3	1.7	1.6	1.0	4.4		0.4	1.3	1.4
22:6n-3	ND[b]	ND	11.3	25.4	27.0	14.4	7.7	13.2
Total n-3	11.2	7.8	41.1	40.8	47.9	38.3	23.1	44.8

[a]Unpublished data (Lin, D.S., and Connor, W.E.). [b]Not detected (<0.005%).

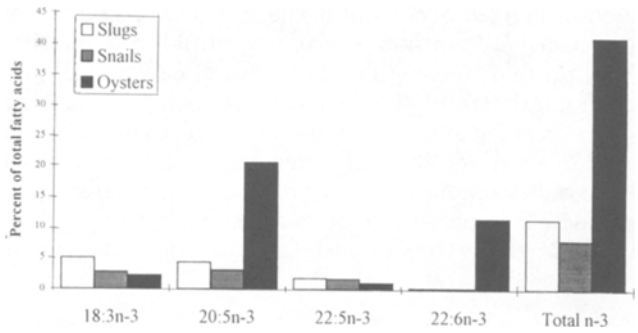

FIG. 1. The n-3 fatty acids of slugs, snails and oysters.

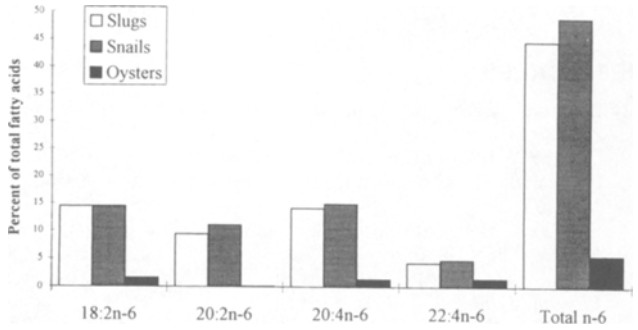

FIG. 2. The n-6 fatty acids of slugs, snails and oysters.

sea-life and is present in high concentration in organs such as brain, retina and sperm of the primates (19,24, 25). The reason for the absence of docosahexaenoic acid in slugs and snails is not known. Long-chain n-3 polyunsaturated fatty acids can be synthesized from dietary linolenic acid (18:3n-3) through a series of desaturation and chain elongation steps. In slugs, the synthesis of the very long-chain and highly polyunsaturated fatty acids proceeded through 20:5n-3 and 22:5n-3, but the further desaturation step to 22:6n-3 did not occur. There was a relative block also for 22:6n-3 synthesis in the mature and full-term human infants fed formulas containing linolenic acid (18:3n-3) (26). In that instance, too, synthesis to 22:5n-3 proceeds well, but the final step, the synthesis of 22:6n-3, occurs but seems to proceed very slowly. Even in adult humans, the feeding of flaxseed, a rich source of 18:3n-3, yields within a few days considerable increases in 20:45n-3 and 22:5n-3, but not in 22:6n-3 (Connor, W.E., unpublished observations).

In slugs there was a relatively high content of the n-6 long-chain polyunsaturated fatty acids such as 20:4n-6, 22:4n-6 and 22:5n-6. Slugs can synthesize linoleic acid from acetate (27). The presence of 22:5n-6 in slugs indicated synthesis from dietary linoleic acid (18:2n-6). Since linoleic and linolenic acids share a common enzymatic system for desaturation and elongation, the presence of 22:5n-6, the last elongation and desaturation product from 18:2n-6, and the absence of 22:6n-3, the last elongation and desaturation product from 18:3n-3, in the slugs suggested that the last step of desaturation and elongation seems to favor n-6 fatty acids synthesis. It is commonly accepted that docosahexaenoic acid is formed from the action of a Δ4 desaturase on 22:5n-3, a precursor fatty acid found in both slugs and snails. Since this enzyme has not been actually identified, an alternative pathway for docosahexaenoic acid synthesis was proposed recently by Voss *et al.* (28).

The absence of docosahexaenoic acid and the presence of an analogous n-6 fatty acid (22:5n-6) in both slugs and snails is in contrast to the mammalian brain and retina that show a decided preference for n-3 fatty acids of their cellular membranes (24). Docosahexaenoic acid is the most abundant polyunsaturated fatty acid in these nervous tissues, whereas 22:5n-6 is conspicuous by very low concentrations except under unusual circumstances. If there is a deficiency of n-3 polyunsaturated fatty acids in the diet (i.e., α-linolenic acid), then 22:5n-6 becomes the predominate fatty acid replacing 22:6n-3 (19,24). The brain of the newborn rabbit also seems to show a preference for 22:5n-6 (29). Since neither 22:6n-3 or 22:5n-6 is present in the diet of slugs and snails so far as known, clearly all of the more polyunsaturated and longer chain fatty acids are derived by synthesis from 18:2n-6 and 18:3n-3, which are richly present in the diet. Despite there being no deficiency of n-3 fatty acids in the diet, the synthetic pathway to an n-6, 22-carbon fatty acid is preferred and is even exclusive. In contrast to slugs and snails, the shellfish mollusks from the sea contain docosahexaenoic acid in quantity. This docosahexaenoic acid probably originated from the phytoplankton of their diet which are rich in both eicosapentaenoic acid and docosahexaenoic acid (30).

Although in our analyses of the three species of slugs and three species of snails in the present study there was a consistent absence of docosahexaenoic acid, Fried *et al.* (12) did find a very small quantity of docosahexaenoic acid (less than 1%), in *Helisoma trivolvis*. Furthermore, free sterols and phospholipids were the two major lipid components in the snails and slugs of this study. Fried *et al.* (10,11) found free sterols and triglycerides were the predominant lipids in *H. trivolvis* snails and *Succinea ovalis* snails. Since analytical methodology was similar between these authors and ours, the difference in results must have occurred from the different species of snails analyzed.

Of the eight sterols found in the slugs, cholesterol was the predominant sterol (76–85% of total sterols) in slugs. This cholesterol could come from dietary intake or *de novo* synthesis. However, slugs are vegetarians and do not consume cholesterol. It is not known whether slugs can synthesize cholesterol from acetate or from the other sterols present in the diet. Among the invertebrates, insects and crustaceans were found unable to synthesize cholesterol from acetate (31). The finding of lathosterol in slugs, and intermediate precursor of cholesterol biosynthesis, suggests the possibility that slugs may be able to synthesize cholesterol. However, direct evidence is needed to verify this point. Interestingly, slug lipids contained significant amounts of brassicasterol (4–13% of total sterols). A high content of brassicasterol was also found in shellfish and phytoplankton. However, the diet of slugs provided abundant plant sterols such as campesterol, sitosterol and stigmasterol

and could provide some other sterols such as brassicasterol. In slug lipids, sterols of possible plant origin contributed only 3–5% of the total sterols. Structurally, brassicasterol is similar to campesterol with one more double bond, at the 22 position. However, these two sterols have an opposite configuration at the 24 position. Brassicasterol differs from stigmasterol only by having a methyl group at the 24 position instead of an ethyl group. In view of this similarity of structure between brassicasterol and the other plant sterols, the high content of brassicasterol found in the slugs may result from dietary sources or from its conversion from dietary plant sterols. In a previous study, we demonstrated that there was dealkylation of sitosterol to form cholesterol in Florida land crab (32).

The results of these analyses provided more detailed information about the sterol and fatty acid composition of the snails although some information is available in the literature (3–12). Similar to our results, Furlong and Caulfield (9) found that cholesterol is the major sterol in the snails. They also found desmosterol, campesterol, stigmasterol and sitosterol in the hepatopancreas of snails. In our study, we found five additional sterols—cholestanol, brassicasterol, lathosterol, 24-methylene cholesterol and sitostanol—in snails. The difference in our two studies may be due to different species of snails analyzed or the higher sensitivity and resolution power of the capillary gas–liquid chromatography we used for our analyses. Snails had the same sterol composition as earthworms (*Lumbricus terrestis*) (33). Slugs had a sterol composition similar to snails and earthworms except for the absence of cholestanol and desmosterol. The reason for these differences is not known. The finding of a high content of desmosterol in snails, (9.1% in *V. columbiana*) and in the earthworm (8.6%) is interesting because usually only trace amounts of desmosterol are present in tissues. Recently, we found a large quantity of desmosterol in the sperm of primates (25). The presence of considerable but variable quantities of desmosterol in the sterols of snails suggest an active cholesterol biosynthesis. While it is true that desmosterol is found in a very limited number of plants, these are plants of the sea such as red algae (34). Desmosterol has not been identified in land plants which constitute the diet of the snails. It seems likely then that snails, in particular, must have the capacity to synthesize cholesterol. Both slugs and snails have a very different sterol composition from the marine mollusks, which contain other unique sterols such as a C_{26} sterol and 22-dehydrocholesterol (1). It is likely that these shellfish obtain their sterols form their diet of phytoplankton (35).

In conclusion, slugs which have evolved from snails (15) have sterol and fatty acid compositions similar but not identical to snails. Relatively high amounts of two biologically important fatty acids, arachidonic and eicosapentaenoic acids, were found in both the slugs and snails. Docosahexaenoic acid (22:6n-3) was completely absent in both slugs and snails. If the n-3 and n-6 fatty acid share the same enzyme system for elongation and desaturation, it is not clear why there was such a complete preference for the synthesis of 22:5n-6 over 22:6n-3. The sterol content of these two land mollusks is quantitatively similar to ocean shellfish but qualitatively quite different. The presence of cholesterol precursors, lathosterol and desmosterol, in snails and lathosterol in slugs suggests that cholesterol synthesis may occur in these mollusks. In view of the unique absence of docosahexaenoic acid and presence of lathosterol and desmosterol, snails and slugs offer fascinating models for the further study of both fatty acid and sterol synthesis.

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Peroxidation Reactions in Plant Membranes: Effects of Free Fatty Acids

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Free fatty acids accumulate in plant membranes after exposure of plants to environmental stress, such as freezing and desiccation. Fatty acid accumulation has been linked to various biophysical changes and to the occurrence of lipid peroxidation, but the relationships appear complex and inconsistent. The interactions between oxygen free radicals, free fatty acids and lipid peroxidation in plant membranes were examined further by studying peroxidation reactions in a model membrane system composed of a complex mixture of plant phospholipids, including various free fatty acids. Multilamellar liposomes were treated with oxygen free radicals generated from iron ascorbate. Increased concentrations of free palmitic acid up to 10 mol% (fatty acid/phospholipid) reduced the production of aldehydes detected by the thiobarbituric acid assay, but enhanced the production of fluorescent products. By contrast, increased concentrations of free linolenic acid increased aldehyde production and reduced the formation of fluorescent products. The two free fatty acids both enhanced the susceptibility of phospholipids to degradation as shown by the reduced recovery of esterified polyunsaturated fatty acids (linoleic and linolenic). The free radical reactions with or without free fatty acid additions catalyzed the selective degradation of phospholipids in the order phosphatidylethanolamine > phosphatidylcholine > phosphatidylinositol > phosphatidylglycerol. Selective degradation of phospholipids is often observed after periods of environmental stress or during senescence of plants, and has been cited as evidence for the involvement of phospholipases in these degenerative processes. The results indicate that selectivity is not a criterion for eliminating the involvement of oxygen free radicals in these degenerative processes. Furthermore, the results suggest that modifications of lipid composition during a plant's acclimation to adverse environments may determine the types of free radical reactions that occur due to stress.

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In plants, increased production of oxygen free radicals and the accumulation of lipid peroxidation products have been associated with a variety of environmental types of stress and with various developmental processes which involve membrane catabolism. For example, lethal freeze–thaw stress of winter wheat has been shown to produce superoxide in a microsomal membrane fraction, promote loss of microsomal lipid-P, increase free fatty acids and increase lipid phase transition temperatures (1,2). Similarly, germinating soybean seeds which had lost desiccation tolerance showed similar physical and compositional changes in a microsomal membrane fraction after drying (3). Plants chilled in high light produced superoxide and singlet oxygen which initiated lipid peroxidation reactions in thylakoids (4). In developmental processes such as senescence, oxygen free radicals have been shown to mediate degradative reactions leading to selective catabolism of phospholipids and formation of gel phase domains (5). The accumulation of free fatty acids in stressed plant tissue can be very high, and values of 20% relative to phospholipid have been reported in aged pollen samples (6).

The peroxidation reactions that occur in plant membranes, however, do not follow the classical reactions which have been detailed so well in model lipid systems (7). For example, treatment of isolated microsomal membranes from winter wheat crowns, or from soybean seeds, with superoxide *in vitro* initiated phospholipid degradation but did not cause a selective degradation of polyunsaturated fatty acids (PUFA) (2,8,9). Under these circumstances, superoxide-initiated deacylation reactions lead to the accumulation of free fatty acids (2,3). The tertiary carbon of the glycerol backbone of phospholipids has been proposed to be the predominant site of free radical attack leading to the deacylation of the phospholipid and accumulation of free fatty acids (10).

Alternatively, phospholipid deacylation and free fatty acid accumulation may be a consequence of enhanced phospholipase activity. This has been the classical interpretation of the results of several studies on membrane catabolism during environmental stress and senescence (11–13). Regardless of whether free fatty acids are produced by oxygen-based radical reactions or by phospholipases, the accumulation of free fatty acids in the membrane bilayer has been linked to a variety of biophysical changes, such as altered lipid microviscosity, elevated lipid phase transition temperatures, and the formation of gel phase domains (14,15). Free fatty acids may also act as fusogens (16–18), promoting membrane fusion within injured cells. Many environmental stresses involve changes in the water status of the plant and in the hydration level of the membrane; two notable examples are desiccation and freezing (19). Sucrose and trehalose are effective membrane-stabilizing agents which prevent the formation of hexagonal and gel phases at low levels of hydration in both model and biological membranes (20). However, if the bilayer contains even trace amounts of free palmitic acid, the stabilizing effect of the sugar is reduced (21). It is also interesting to note that perturbed or destabilized membrane bilayers, such as those containing free fatty acids, are more susceptible to further degradation (5).

Thus, oxygen-based free radicals, phospholipid catabolism, lipid peroxidation and free fatty acid accumulation seem to play a common role in membrane catabolism during senescence or environmental stress, and these factors interact in a complex manner that cannot be fully explained based on current knowledge of free radical reactions in plant membranes.

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

In the present study, the effects of free fatty acids on both the susceptibility of plant membrane phospholipids to lipid peroxidation, as well as the types of degradative products formed in a model membrane system have been investigated in an attempt to better understand these interactions. Liposomes composed of soybean asolecithin, a complex mixture of plant phospholipids, and various free fatty acids were exposed to free radicals generated by Fe^{3+}, ethylenediaminetetraacetic acid (EDTA) and ascorbate, which is a variation on the Fenton reaction producing hydroxyl, perferryl and superoxide radicals.

MATERIALS AND METHODS

Treatment of model membranes. Soybean asolecithin (Fluka, Buchs, Switzerland) was dissolved in chloroform (30 mg/mL), bound to a sep-pak silica column and sequentially eluted with 10 mL chloroform, 20 mL acetone and 20 mL methanol. Only the methanol fraction containing the purified phospholipids was used to prepare liposomes. Free fatty acids (Sigma, St. Louis, MO) were added, without further purification, to the methanolic phospholipid fraction prior to formation of the multilamellar liposomes. In most experiments, free fatty acids were added to the phospholipids to a final concentration of 0, 2.5, 5 or 10 mol% (fatty acid/phospholipid) in chloroform. This fraction was dried under vacuum, resuspended in 10 mM KH_2PO_4 (pH 6.8) buffer, and vortexed to form multilamellar liposomes.

To treat the liposomes with oxygen free radicals, 2-mL samples of the liposomes were added to 2 mL 10 mM KH_2PO_4 buffer, 0.5 mL 10 mM Fe^{2+}–EDTA and 0.5 mL 20 mM ascorbate as described previously (9). Because the free radical reactions proceeded so quickly, the time 0 samples were taken without the addition of the free radical generating system, but simply with the addition of phosphate buffer. Treated samples of 0.5 mL were removed in most experiments at 30, 60, 90 and 120 min. The reaction was stopped by the immediate addition of either the chemical reagents for a specific assay or by chloroform/methanol addition.

Lipid analysis. To analyze the lipid composition of the initial or the free radical-treated liposome preparations, liposomes were partitioned against two volumes of chloroform/methanol (1:1, vol/vol). The extracted phospholipids were separated by thin-layer chromatography using a solvent system consisting of chloroform/methanol/water (65:35:5, by vol). The phospholipids were extracted from the silica with chloroform/methanol (1:1, vol/vol), dried, and resuspended in 4 mL methanolic KOH, capped under nitrogen, and heated at 70°C for 15 min for transmethylation (9). Diheptadecanoyl phosphatidylcholine was added prior to transmethylation to serve as an internal standard in phospholipid quantitation.

To assay aldehyde reaction products, 2 mL thiobarbituric acid (TBA) reagent (0.375% TBA, 15% trichloroacetic acid) was added to the reaction mixture, immediately heated at 100°C for 15 min, cooled, and a glacial acetic acid/chloroform (1:3, vol/vol) mixture was added to clear the emulsion. Absorbance of the aqueous layer was measured at 532 nm. To quantify hydroperoxides, the same procedure was followed except that 25 μL of 1 g of butylated hydroxytoluene (BHT) in 2 mL acetone was added to the TBA reagent (22). Fluorescent peroxidation products were quantified in the reaction solutions by scanning at an excitation wavelength of 360 nm and an emission range of 370–550 nm (23).

Experimental design and statistical analysis. Multilamellar liposomes were formed from mixtures of phospholipid and various free fatty acids. The free fatty acid concentrations chosen were intended to span the concentrations observed previously in plant microsomal membranes (1–3,5,6), but based on the preliminary experiments, we chose only one unsaturated (linolenic acid) and one saturated (palmitic acid) fatty acid. The liposomes were treated in aqueous solution with oxygen free radicals from a mixture of iron ascorbate, and the products were usually measured at 2 h after the addition of the iron.

All experiments were conducted in a randomized complete block design replicated at least three times over time. The data for thiobarbituric acid reactive substances (TBARS) were statistically evaluated by analysis of variance using a two-way factorial design. The data were also analyzed as a power regression:

$$y = ax^b \ (a > 0) \qquad (1)$$

All other experiments were statistically analyzed using a pooled *t*-test for comparison of the free fatty acid treatments to the control at 95% confidence levels.

RESULTS

The soybean asolecithin used in this study to prepare the liposomes was composed primarily of four phospholipids, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG) at concentrations of 44, 22, 18 and 11% of the total phospholipids, respectively (Table 1). Phosphatidic acid (PA) was found to be present in quantities of 6% of the total phospholipid fraction. The fatty acid composition of each of the phospholipid classes was similar for PC, PI, PG and PE. Linoleic and palmitic acids were the predominant fatty acids in each of these phospholipids. PA was the exception, with higher levels of linolenic acid and lower levels of linoleic acid than the others. The preparation is similar in composition to lipid extracts from microsomal membranes of seed and cotyledon tissues (3,6,8), and

TABLE 1

Composition of Soybean Asolecithin Used to Prepare Liposomes Prior to Free Radical Treatment[a]

Phospholipid	Proportion of total phospholipids (%)	Fatty acid (mol%)				
		16:0	18:0	18:1	18:2	18:3
PA	6	26	16	11	23	25
PC	44	20	7	9	55	9
PI	18	27	7	8	55	14
PG	11	27	9	8	43	13
PE	22	22	8	8	49	13

[a]PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.

represented a convenient means of modelling the effects of free fatty acids in a plant membrane.

The addition of various free fatty acids to the soybean asolecithin model membrane system altered the free radical reactions according to a number of assays measuring both the products and fatty acid substrates of the peroxidation reaction.

The TBA assay typically measures a variety of aldehyde products produced in lipid peroxidation (22,23). TBARS rapidly increased upon treatment of the soybean asolecithin vesicles with free radicals. Aldehyde product formation increased rapidly in the first 30 min of the reaction and more slowly later. The addition of free palmitic acid to the soybean asolecithin vesicles significantly ($P = 0.05$) decreased TBARS accumulation (Table 2). For example, after 2 h the TBARS seen in liposomes containing 10 mol% free palmitic acid were reduced to approximately 50% compared to those seen in liposomes without free fatty acid addition. In contrast, addition of free linolenic acid significantly ($P = 0.05$) increased TBARS at all reaction times. For example, liposomes containing 5 mol% free linolenic acid after 2 h free radical treatment had TBARS values of 169% relative to that of the control (Table 3).

The kinetics of TBARS formation in these reactions were fitted to the power regression equation, and all free fatty acid additions had r^2 values of not less than 0.98, with the exception of 5 mol% free linolenic acid ($r^2 = 0.89$). Therefore, there did not seem to be major changes in the reaction kinetics with the addition of the free fatty acids, indicating that the reactions were uniformly promoted or inhibited at all sampling times.

The addition of free stearic acid to the soybean asolecithin liposomes had no effect on the formation of TBARS as a result of free radical treatment (data not shown). Therefore, the addition of free fatty acids to the soybean asolecithin increased, decreased or had no effect on the formation of aldehydes during free radical mediated peroxidation, depending upon the type of free fatty acid added to the liposome. This phenomenon was followed in more detail using two of the free fatty acids which differed most dramatically in their effects, namely palmitic and linolenic acids.

Peroxidation reactions also produce hydroperoxides, which can react in the TBA assay. Adding BHT to the TBA assay prevents hydroperoxides from contributing to the estimation of TBARS (22). The TBA assay measured all aldehyde peroxidation products, TBA with BHT measured the aldehyde products excluding the hydroperoxides, and the difference between the two assays gave an indication of the amount of hydroperoxides produced. In the liposomes without free fatty acid addition, relatively low levels of hydroperoxides were present after 2 h of reaction (Table 4), presumably because they were consumed in reactions with Fe^{2+}–EDTA. Increasing mol% of free palmitic acid in the liposomes reduced hydroperoxides, but this was not statistically significant because of the low initial values. With addition of free linolenic acid to the soybean asolecithin liposomes, greater amounts of hydroperoxides were formed at increasing concentrations of free linolenic acid.

Schiff-base structures, formed from the reaction of malondialdehyde (MDA) and other aldehydes with amino groups, are fluorescent products of the peroxidation reaction (23). The addition of 2.5 and 5 mol% free palmitic acid produced significantly ($P = 0.05$) greater concentrations of fluorescent products (Table 5). However, no significant differences were noted in the fluorescent intensities among the three different free fatty acid concentrations. With addition of free linolenic acid to the soybean asolecithin liposomes, lesser amounts of fluorescent peroxidation products were produced. The formation of these products in soybean asolecithin liposomes increased with reaction time up to 2 h following the power regression with r^2 val-

TABLE 2

Thiobarbituric Acid Reactive Substances in Soybean Asolecithin Liposomes Containing Free Palmitic Acid After Exposure to Free Radicals[a]

Time (min)	Free fatty acid additions (mol%)			
	0	2.5	5	10
0	0	0	0	0
30	0.15 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.08 ± 0.01
60	0.21 ± 0.04	0.16 ± 0.01	0.15 ± 0.03	0.11 ± 0.01
90	0.23 ± 0.05	0.18 ± 0.02	0.17 ± 0.03	0.12 ± 0.01
120	0.25 ± 0.04	0.19 ± 0.02	0.18 ± 0.05	0.12 ± 0.02

[a]Values represent the absorbance at 532 nm (±SD) derived from three determinations.

TABLE 3

Thiobarbituric Acid Reactive Substances in Soybean Asolecithin Liposomes Containing Free Linolenic Acid After Exposure to Free Radicals[a]

Time (min)	Free fatty acid (mol%)		
	0	2.5	5
0	0	0	0
30	0.15 ± 0.02	0.15 ± 0.07	0.24 ± 0.08
60	0.21 ± 0.04	0.21 ± 0.11	0.27 ± 0.15
90	0.23 ± 0.05	0.29 ± 0.04	0.36 ± 1.09
120	0.25 ± 0.04	0.32 ± 0.06	0.44 ± 0.13

[a]Values represent the absorbance at 532 nm (±SD) derived from three determinations.

TABLE 4

Thiobarbituric Acid Reactive Substances (TBARS) in Soybean Asolecithin Liposomes Containing Free Palmitic or Linolenic Acids After Two Hours of Exposure to Free Radicals[a]

Free fatty acid	mol%	TBARS	TBARS + BHT	Hydroperoxides
Control	0	0.23 ± 0.04	0.17 ± 0.01	0.06 ± 0.05
Palmitic	2.5	0.19 ± 0.02	0.13 ± 0.01	0.06 ± 0.03
	5	0.18 ± 0.05	0.14 ± 0.02	0.04 ± 0.07
	10	0.12 ± 0.02	0.15 ± 0.03	0.03 ± 0.05
Linolenic	2.5	0.32 ± 0.06	0.20 ± 0.04	0.12 ± 0.10
	5	0.44 ± 0.13	0.16 ± 0.01	0.28 ± 0.14

[a]Values represent the absorbance at 532 nm (±SD) derived from three determinations. BHT, butylated hydroxytoluene.

TABLE 5

Fluorescent Compounds Formed in Soybean Asolecithin Liposomes Containing Free Palmitic or Linolenic Acids After Exposure to Free Radicals[a]

Free fatty acid	mol%	Time of free radical exposure (min) 0	30	60	90	120
Control	0	4.58	7.11	8.22	9.38	10.38
Palmitic	2.5	3.18	6.38	9.33	12.13[b]	14.93[b]
	5	3.35	6.68	10.38[b]	13.79[b]	17.73[b]
	10	2.30	5.85	9.50[b]	12.30[b]	14.14[b]
Linolenic	2.5	6.53	6.35	7.49	8.25	9.15
	5	6.00	5.57[b]	6.70	7.67	8.10
	10	4.08	3.47[b]	4.60[b]	5.25[b]	5.92[b]

[a]Values are expressed as relative fluorescent intensity.
[b]Denotes a significant difference between treatment and control at the 95% confidence level.

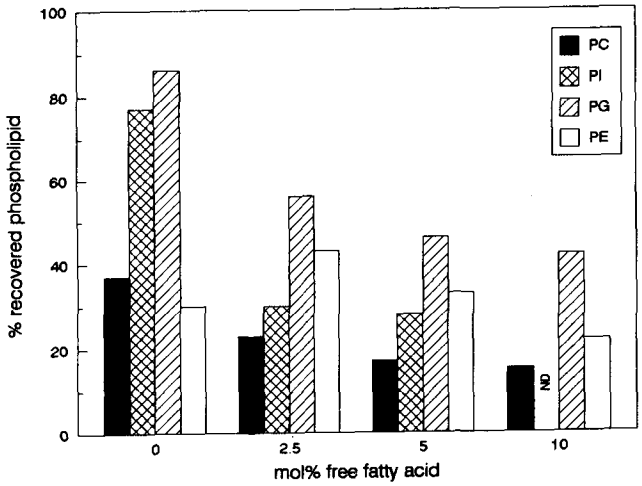

FIG. 1. Recovery of individual phospholipids after Fe-ascorbate treatment of multilamellar liposomes containing varying amounts of free palmitic acid. Percent recovery measures the quantity of a phospholipid after treatment (t = 2 h) relative to the quantity before treatment (t = 0 h). ND, not detected. PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.

ues of not less than 0.99, with the exception of 5% free linolenic acid ($r^2 = 0.94$). This indicates that like the formation of TBARS, the reaction kinetics for the formation of fluorescent products were not changed with the addition of free fatty acids, and that the reactions were uniformly promoted or inhibited at all sampling times.

The major source of substrate for the free radical-mediated peroxidation reactions is the esterified PUFA in the phospholipids of the soybean asolecithin liposomes. Only 56% of all esterified fatty acids were recovered after the 2-h free radical exposure, indicating that 44% of the phospholipids had been degraded. The proportion of PUFA in the esterified fatty acid fraction decreased from 67 to 48% (Table 6). This seems to be the result of the preferential degradation of PUFA and the proportional increase in saturated fatty acids. With the addition of either palmitic or linolenic acid, the degradation of all phospholipids increased (data not shown), and the selective degradation of PUFA increased slightly, reducing the proportion of PUFA to approximately 32% with the addition of 10 mol% of either free fatty acid.

Without the addition of free fatty acids, there was selective degradation of specific phospholipid classes during the free radical-mediated peroxidation reactions. After 2 h incubation, 30% of PE, 38% of PC, 78% of PI and 88% of PG were recovered. The addition of free palmitic acid or free linolenic acid enhanced the degradation of PC, PG and PI, but had relatively little effect on PE (Figs. 1 and 2).

The recovery of esterified PUFA in each phospholipid class followed a similar pattern. After 2 h of free radical exposure, 77% of the PUFA from PE and 60% from PC were degraded, but the PUFA in PI and PG were rela-

TABLE 6

Proportion (mol%) of the Esterified Fatty Acids Recovered as Polyunsaturated Fatty Acids (PUFA) from Soybean Asolecithin Liposomes Containing Free Palmitic or Linolenic Acids After Exposure to Free Radicals[a]

mol% added	mol% PUFA Palmitic	Linolenic
0	48 ± 8.0	48 ± 8.0
2.5	37 ± 0.8	43 ± 6.1
5	37 ± 5.4	33 ± 4.9
10	32 ± 12.3	32 ± 6.5

[a]Values represent the means of linoleic and linolenic acids relative to all esterified fatty acids (±SD) derived from three determinations. The proportion of PUFA in the liposomes prior to free radical treatment averaged 67%.

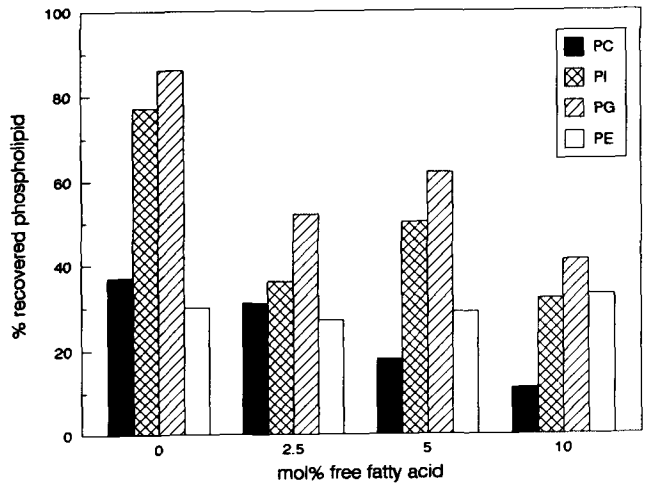

FIG. 2. Recovery of individual phospholipids after Fe-ascorbate treatment of multilamellar liposomes containing varying amounts of free linolenic acid. Percent recovery measures the quantity of a phospholipid after treatment (t = 2 h) relative to the quantity before treatment (t = 0 h). Abbreviations as in Figure 1.

TABLE 7

Recovery of Esterified Polyunsaturated Fatty Acids of the Four Major Phospholipid Classes of Soybean Asolecithin After Two Hours of Treatment with Free Radicals[a]

Free fatty acid	mol%	% Recovery			
		PE	PC	PI	PG
Control	0	23	40	73	100
Palmitic	2.5	19	18	27	43
	5	21	14	54	35
	10	15	13	ND	30
Linolenic	2.5	20	37	44	80
	5	28	15	43	39
	10	22	8	25	29

[a]Values represent nmol PUFA recovered after free radical treatment/nmol PUFA without free radical treatment × 100%. Abbreviations as in Tables 1 and 6.

tively more resistant to degradation (Table 7). All concentrations of free palmitic acid and free linolenic acid significantly amplified the loss in esterified PUFA.

DISCUSSION

In the model membrane system of soybean asolecithin liposomes, addition of free fatty acids increased the susceptibility of the lipids to free radical-mediated degradation as shown by reduced recovery of PUFA and phospholipid. However, the type of free fatty acid present affected the types of peroxidation products formed. Increasing concentrations of free palmitic acid in the liposomes increased formation of fluorescent peroxidation products, but reduced hydroperoxides and TBARS. In contrast, increasing concentrations of free linolenic acid in the liposomes caused no change to the amount of fluorescent peroxidation products, and increased hydroperoxides and TBARS.

These differences in the type of peroxidation product formed as a result of the inclusion of different free fatty acids in the liposome may indicate that the free fatty acid was acting either chemically as a substrate in the peroxidation reactions, or physically as a perturbing agent altering the packing, fluidity, and/or physical arrangement of the phospholipids in the multilamellar liposome. Free linolenic acid containing three double bonds is highly susceptible to free radical attack. Thus, 18:3 is likely a substrate in the peroxidation reactions resulting in the formation of TBARS and hydroperoxides from the direct peroxidation of the free fatty acid itself. On the other hand, free palmitic acid containing no double bond is not an immediate substrate in peroxidation reactions.

The liposomes used in this study were multilamellar, and the radicals were generated in the aqueous compartment outside the liposome. Unilamellar vesicles have been shown to react to this free radical treatment differently than multilamellar vesicles (9). It is therefore conceivable that the presence of the free fatty acids altered fluidity (14), thereby changing the susceptibility of lipids to the free radical reactions. These changes are perhaps associated with the closer apposition of the fatty acid chains imposed by the presence of free palmitic acid.

Furthermore, compounds such as dihydropyridines may be formed by reaction of MDA with amines, such as ethanolamine, producing fluorescent structures (23). Of the individual classes of phospholipids, PE was most sensitive to the peroxidation reaction, especially in the absence of free fatty acids. Therefore, it is likely that a considerable proportion of the fluorescent peroxidation products was Schiff-base structures formed by reaction of the amine group of PE with aldehyde peroxidation products. However, as more free palmitic acid was added, the production of fluorescent products increased but the extent of PE degradation did not. In this instance, the rise in fluorescent intensity noted may be solely due to the production of fluorescent aldehyde polymers.

The selective degradation of specific phospholipid classes has been observed in several systems undergoing membrane catabolism, and has since been attributed to the selectivity of the phospholipase degradation (11–13). In the soybean asolecithin liposomes, sensitivity to degradation by the free radical reactions followed the order PE > PC > PI > PG. The differences in susceptibility are not due to differences in the proportion of PUFA present in each phospholipid class because their initial PUFA composition ranged from only 69 to 56% and followed the order PI > PC > PE > PG. The involvement of PE in the formation of Schiff-base structures may explain its greater sensitivity to degradation. Differences in molecular species and the relative lateral distribution of the phospholipids in the liposome may influence exposure of the individual PUFA to the free radical reactions. The selective loss of PC has been seen in tissues of nonhardy plants exposed to freezing temperatures (11), as well as in plants during senescence (12,13). In both cases, degradation was attributed to increased phospholipase activity. In the model membranes used in this study, no proteins or enzymes were present, simply lipid, Fe and ascorbate. Therefore, the selective degradation of phospholipid can be attributed solely to free radical reactions. Consequently, selective degradation of phospholipid is not a good criterion for phospholipase and other hydrolytic enzyme activities, especially in plant cells under conditions which are known to highly favor free radical reactions such as freezing (2) and senescence (5).

In summary, the presence of free fatty acids in a model membrane increased lipid degradation and altered the formation of peroxidation products. Therefore, the accumulation of free fatty acids in plant cell membranes during periods of environmental stress, whether the result of phospholipase activity or free radical mediated deacylation, would be expected to have a number of deleterious consequences, but the effect on the membrane will differ depending on the type of free fatty acid that accumulates. Therefore, the initial fatty acid composition of the membrane is very important in determining the membrane's response, not only because it will influence its susceptibility to free radical attack, but also because it will determine the type of free fatty acid liberated by deacylation reactions. Furthermore, the phospholipid headgroup appears to play a significant role in determining a phospho-

lipid's susceptibility to free radical attack. During acclimation to environmental stress, plants alter the lipid composition of their membranes (24). This has been interpreted almost exclusively in terms of membrane fluidity and lipid phase changes (24). Acclimation also causes the membranes and plants to become increasingly resistant to free radical attack (2,25). The degree to which changes in fatty acid unsaturation and in phospholipid class are responsible for this difference in resistance has yet to be determined.

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METHOD

Reactions of Diazomethane with Glycerolipids in the Presence of Serum or Inorganic Salts[1]

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Diazomethane is widely used for the selective methylation of nonesterified fatty acids in the presence of other lipids. However, when the reaction is carried out directly with plasma or serum, substantial methanolysis of phospholipid acyl groups occurs. Because of the importance of rigorous selectivity in the assay of unesterified fatty acids which are present only in trace amounts in cells and body fluids, we have investigated the diazomethane procedure in detail and reached the following conclusions: (i) When diazomethane reacts with lipid extracts in organic solvent, no ester hydrolysis occurs. (ii) In the presence of serum or plasma, diazomethane reacts with water and inorganic salts, causing the solution to become basic ($CH_2N_2 + NaCl + HOH \rightarrow CH_3Cl + Na^+ + OH^- + N_2$); methoxide ions are formed from methanol ($CH_3OH + OH^- \rightarrow CH_3O^- + HOH$) causing extensive methanolysis ($CH_3O^- + RO\text{–}CO\text{–}R' \rightarrow CH_3O\text{–}CO\text{–}R' + RO^-$). An analogous reaction takes place with ethanol. All esters of glycerol are transesterified in aqueous salt solution by this mechanism. It is therefore essential to prepare a lipid extract prior to the assay of unesterified fatty acids when using the diazomethane procedure.

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Diazomethane is an extremely versatile reagent that has been used in synthetic organic chemistry for more than a century (1–3). In the lipid field, diazomethane is employed for the selective methylation of unesterified fatty acids (4) in the presence of other lipids. We have used this method extensively over the past 15 years (5–9). In the usual procedure, a dilute solution of diazomethane in diethyl ether is added to a lipid extract dissolved in diethyl ether/methanol (9:1, vol/vol). It has been shown that under these conditions the methyl group of the fatty acid methyl ester is derived from diazomethane (see Equation 1). The methanol in the solvent does not participate in the reaction (4). Experiments with ^{18}O-labeled fatty acids have shown that both fatty acid oxygens are retained in the methyl ester (6).

$$R\text{–}COOH + CH_2N_2 \rightarrow R\text{–}COOCH_3 + N_2 \qquad [1]$$

In a method developed for the rapid estimation of unesterified fatty acids in human blood plasma through "extractive methylation," Pace-Asciak (10) added ethereal diazomethane directly to a 1:1 (vol/vol) mixture of plasma and methanol. However, Lin *et al.* (11) later reported that diazomethane caused extensive methanolysis of phospholipid acyl groups under the conditions described by Pace-Asciak (10), and they questioned the selectivity of fatty acid methylation by diazomethane in the presence of other lipids. Unfortunately, Lin *et al.* (11) did not offer a hypothesis regarding the mechanism of the reaction and certain ambiguities in the reported experimental details could lead to confusion about the selectivity of the diazomethane reaction in organic solvent as well. Because of the importance of such selectivity for the methylation of unesterified fatty acids, which are often present only in trace amounts, and because we have never observed (5–9) transesterification or hydrolysis of ester bonds induced by diazomethane, we investigated possible causes for the results reported by Lin *et al.* (11).

The data presented here confirm the conclusions of Schlenk and Gellerman (4) that diazomethane does not react with lipid ester bonds in organic solvents. However, we found that diazomethane in the presence of water, inorganic salts and methanol causes extensive methanolysis of glycerolipids. The same sequence of reactions occurs if plasma is substituted for the inorganic salt solution.

MATERIALS AND METHODS

Diazald™ (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide), dipalmitoyl phosphatidylethanolamine (DPPE) and dimyristoyl phosphatidylcholine (DMPC) were from Sigma Chemical Company (St. Louis, MO). 1-Palmitoyl lysophosphatidylcholine and 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) were from Avanti Polar Lipids (Birmingham, AL). Heptadecanoic acid, methyl heptadecanoate, arachidonic acid, cholesteryl oleate (CE) and tripentadecanoylglycerol (TAG) were from Nu-Chek-Prep (Elysian, MN).

Diazomethane was prepared from Diazald essentially as described by Schlenk and Gellerman (4) and as used routinely in our laboratory (5–9). The diazomethane produced from one gram of Diazald was recovered in approximately 30 mL of diethyl ether to give a solution with an estimated concentration of less than 150 mM. This solution was stored in tubes with Teflon-lined screw caps at –20°C for up to three weeks. All operations that necessitated opening a vessel containing diazomethane were conducted in a fume hood. Diethyl ether, containing ethanol as a preservative, was freshly distilled in an all-glass system before use. Citrated plasma was prepared from blood drawn from a healthy, nonfasting volunteer.

Methanolysis/methylation in plasma.[2] In the procedure described by Pace-Asciak (10), 0.2 mL of plasma is mixed

[1]We dedicate this paper to Dr. Hermann Schlenk on the occasion of his 80th birthday.

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Abbreviations: BSA, bovine serum albumin; CE, cholesteryl ester (cholesteryl oleate); DMPC, dimyristoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; GC, gas chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LPC, lysophosphatidylcholine (1-palmitoyl lysophosphatidylcholine); SOPC, 1-stearoyl-2-oleoyl phosphatidylcholine; TAG, triacylglycerol (tripentadecanoylglycerol); TLC, thin-layer chromatography.

[2]Methylation is defined as the addition of a methyl group from diazomethane to an unesterified fatty acid; methanolysis (or transesterification) means alcoholysis with methanol, in which methoxide ion reacts with a glycerol ester to form a methyl ester.

with 0.2 mL of methanol, 12 μg of pentadecanoic acid and 1 mL of ethereal diazomethane (derived from about 0.1 g of Diazald). After 10 min at room temperature, an 800 μL aliquot of the upper (diethyl ether) phase is transferred to another tube, taken to dryness and the residue is dissolved in hexane for analysis by gas chromatography (10).

We used 10 μg of heptadecanoic acid as internal standard. In order to conserve reagents, our standard reaction mixture usually consisted of 0.1 mL of plasma (or salt solution), 0.1 mL of methanol and 0.5 mL of ethereal diazomethane. Samples were vortexed and then reacted for 10 min at room temperature in open tubes in a fume hood. The diethyl ether and residual diazomethane were quickly removed under a stream of nitrogen, and the methyl esters were extracted from the aqueous phase into 5 mL of hexane/diethyl ether (4:1, vol/vol) or into 2 mL of chloroform/methanol (2:1, vol/vol).

In later experiments the volume of methanol was increased to 0.3 mL to form a single phase with the ether and water.

Lipid extraction before methylation. Plasma aliquots of 0.1 mL were mixed with 10 μg of heptadecanoic acid as internal standard, extracted with 4 mL of chloroform/methanol (2:1, vol/vol) and partitioned with 1 mL of 2.5% NaCl in small, screw-cap test tubes (12). The tubes were centrifuged, the organic layer was removed and blown to dryness under nitrogen. The lipids were redissolved in 1 mL of diethyl ether/methanol (9:1, vol/vol), and 0.5 mL of diazomethane was added. After 10 min at room temperature, the diethyl ether and diazomethane were removed under a stream of nitrogen.

Purification of methyl esters. When diazomethane was added to a lipid mixture, the methyl esters were usually purified before gas chromatography (GC). After reaction with diazomethane, the lipids were dissolved in 1 mL of hexane and applied to a small column of silicic acid (about 1 g) in a disposable glass transfer pipette. The test tube was rinsed with an additional 2 mL of hexane that was also applied to the column. Methyl esters were eluted with 4 mL of hexane/diethyl ether (9:1, vol/vol). Cholesterol and polar lipids remained on the column. The elution scheme was checked for recovery of methyl esters by analyzing one of the samples by GC before and after purification.

GC of methyl esters. GC was performed with a Packard Model 428 GC equipped with dual flame-ionization detectors and a Spectra-Physics 4270 integrator. Methyl esters were separated on a 12 ft × 1/8 in. aluminum column packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Bellefonte, PA), with the temperature programmed from 180 to 230°C at a rate of 3°C per min.

RESULTS

Unesterified fatty acids in human plasma. In a direct comparison of the two methods, aliquots of fresh citrated plasma were treated following the procedure of Pace-Asciak (10), or the plasma lipids were extracted before reaction with diazomethane. Each group of assays included six replicates. The results of the GC analyses of methyl esters are given in Table 1. As expected (11), the Pace-Asciak procedure (10) yielded a concentration of methyl esters ten times the amount detected after lipid extraction, with a large standard deviation. There was also a difference in the composition of the methyl esters. Long-chain polyunsaturated fatty acid comprised more than 15% of the fatty acid methyl esters obtained by the Pace-Asciak procedure (10) but were not present in the methyl esters recovered from the lipid extracts. The extracts contained a higher percentage of methyl oleate.

TABLE 1

Comparison of Plasma Unesterified Fatty Acids Determined by the Pace-Asciak Procedure (Ref. 10) and After Extraction[a]

	Pace-Asciak (Ref. 10)		Extract	
	(Methyl esters recovered)			
Fatty acid	μg/mL	wt%	μg/mL	wt%
16:0	76.8 (±22.7)	28.5	7.5 (±0.9)	26.2
16:1	3.7 (±1.5)	1.4	0.6 (±0.2)	2.2
18:0	38.8 (±12.2)	14.4	3.4 (±0.4)	11.9
18:1	50.5 (±12.4)	18.8	12.1 (±1.2)	42.3
18:2n-6	57.7 (±18.4)	21.4	5.0 (±1.0)	17.4
20:3n-6	5.45 (±2.05)	2.0	n.d.	
20:4n-6	23.8 (±8.45)	8.8	n.d.	
22:5n-3	1.45 (±0.70)	0.5	n.d.	
22:6n-3	11.0 (±2.95)	4.1	n.d.	
Total μg/mL	269.0 (±80.6)		28.6 (±3.6)	
μM	960		100	

[a]Aliquots of fresh citrated plasma from a nonfasting volunteer were mixed with methanol and diazomethane/diethyl ether (Ref. 10) or were extracted with chloroform/methanol (2:1, vol/vol) and the lipid extracts were treated with diazomethane (Extract). Methyl esters were quantitated by gas chromatography using an internal standard (heptadecanoic acid, added before extraction or before treatment with diazomethane) as described in the Materials and Methods section. Values are mean ± SD of six replicates in each group. n.d., Not detected.

Because we found only traces of unesterified arachidonic acid in the lipid extracts, whereas it is reported to be a prominent component in other assays (11,13), we checked its recovery by adding known amounts of arachidonic acid to serum before lipid extraction and analysis. As little as 0.7 μg added to 0.1 mL of serum before extraction could be measured with complete recovery (data not shown).

Conditions that affect the methanolysis of complex lipids. Figure 1 shows that the amounts of methyl esters formed by "extractive methylation" (10) increased with time and with increasing concentrations of diazomethane.

In order to determine which components of the mixture are necessary for methanolysis of plasma lipids, 0.1 mL aliquots of plasma were incubated with methanol, methanol plus diethyl ether or ethanol plus diethyl ether for 20 min before lipid extraction. The yield of methyl esters was the same as after extraction alone (Table 2).

Because the reaction was catalyzed by an interaction between diazomethane and one or more of the plasma components, plasma lipids were extracted from 0.1 mL aliquots, redissolved in 0.1 mL methanol and mixed with 0.1 mL of solutions of bovine serum albumin (BSA), BSA in 1 M NaCl or 1 M NaCl. Upon treatment with diazomethane, methanolysis was extensive in the solutions

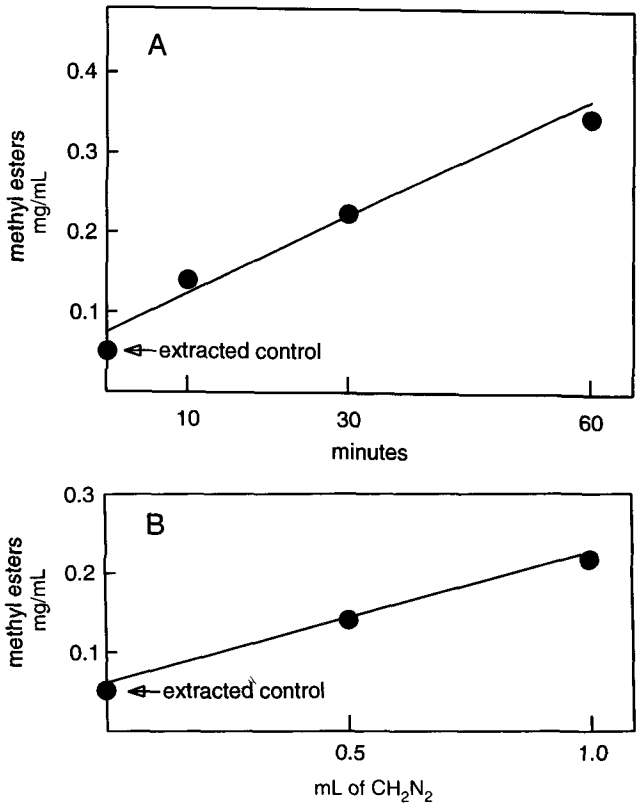

FIG. 1. Plasma (0.1 mL), methanol (0.1 mL) and ethereal diazomethane (0.5 mL) were incubated for different periods of time (A) and for 10 min with different amounts of diazomethane (B). Methyl esters were analyzed as described in the Materials and Methods section.

containing NaCl and did not increase when protein was present. Protein in the absence of NaCl did not cause methanolysis, nor did water alone. NaCl in the absence of diazomethane had no effect (Table 3).

Methanolysis of different lipid classes. In order to quantify the reaction of various lipid classes, we used mixtures of synthetic lipids containing different fatty acids, i.e., CE, TAG, DMPC, 1-palmitoyl lysophosphatidylcholine (LPC),

TABLE 2

Preincubation of Plasma Before Extraction[a]

Treatment (0.1 mL plasma)	Total methyl esters (μg/mL plasma)
Methanol (0.1 mL, 20 min)	30.3
Methanol (0.1 mL) and diethyl ether (0.5 mL, 20 min)	29.0
Ethanol (0.1 mL) and diethyl ether (0.5 mL, 20 min)	28.4
No additions	24.8
Methanol (0.1 mL) and ethereal diazomethane (0.5 mL)	139.1

[a]Plasma was incubated with the indicated additions, followed by extraction with chloroform/methanol (2:1, vol/vol) and reaction with diazomethane as described in the Materials and Methods section. Methyl esters were quantitated by gas chromatography in the presence of an internal standard.

TABLE 3

Methanolysis of Lipids Extracted from 0.1 mL Plasma and Mixed with Protein Solutions Plus Methanol and Ethereal Diazomethane[a]

Addition (0.1 mL)	Methyl esters (μg)
Albumin in 1 M NaCl	50.4
1 M NaCl	69.7
Plasma	34.2
Albumin, no NaCl	7.3
No additions (extracted control)	4.6
1 M NaCl, no diazomethane (extracted)	5.3

[a]Bovine serum albumin was dissolved in 1 M NaCl or in water at a concentration of 70 mg/mL. These solutions were added to the lipid extracts (from 0.1 mL plasma) dissolved in 0.1 mL of methanol. Ethereal diazomethane (0.5 mL) was added and after 10 min the methyl esters were extracted and quantitated by gas chromatography as described in the Materials and Methods section.

SOPC and DPPE. GC analysis of methyl esters in the presence of an internal standard was used to quantify the degree of methanolysis of each lipid class. Mixtures did not include more than one palmitate-containing lipid.

A lipid mixture (CE, TAG, DMPC and LPC) was incubated with diazomethane and several different salt solutions ($CaCl_2$, NaBr, KCl, Na_2SO_4) in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.4. All salts tested were equally effective in promoting the methanolysis of DMPC and LPC, but CE and TAG were not affected. Twenty-five mM HEPES also caused some methanolysis, but water alone did not. There was no difference in the rate of methanolysis between the *sn*-1 and *sn*-2 position of SOPC when incubated with 1 M NaCl. DPPE reacted at about the same rate as DMPC and SOPC (data not shown).

When a mixture of PC, LPC and TAG was reacted with increasing concentrations of NaCl, LPC was preferentially hydrolyzed, DMPC reacted at a slower rate, and TAG was not affected (Fig. 2A). Under these conditions, the reactants formed a two-phase system. However, when the volume of methanol was increased to 0.3 mL, a single phase was formed and all lipids tested, with the exception of cholesteryl esters, were substrates for the reaction (Fig. 2B). The data were explained by considering that in the two-phase system, LPC readily partitions into the aqueous/methanol phase, DMPC partitions less readily, and TAG remains in the ether phase, with the reaction taking place in the aqueous/methanol phase.

In the one-phase system of 0.3 mL methanol, 0.1 mL aqueous NaCl and 0.5 mL ethereal diazomethane, as little as 1 mM NaCl was sufficient to cause measurable methanolysis (data not shown). At 1 M NaCl, methanolysis of DMPC, LPC and TAG was complete.

When ethanol replaced methanol, ethyl esters were produced, proving that the mechanism involves transesterification or alcoholysis and that diazomethane is not directly involved in the methylation. Substituting isopropranol did not result in the production of isopropyl esters, probably for steric reasons, but led to a slight increase in the recovery of methyl esters. Presumably, in this case acyl groups were hydrolyzed due to the increase in pH and the

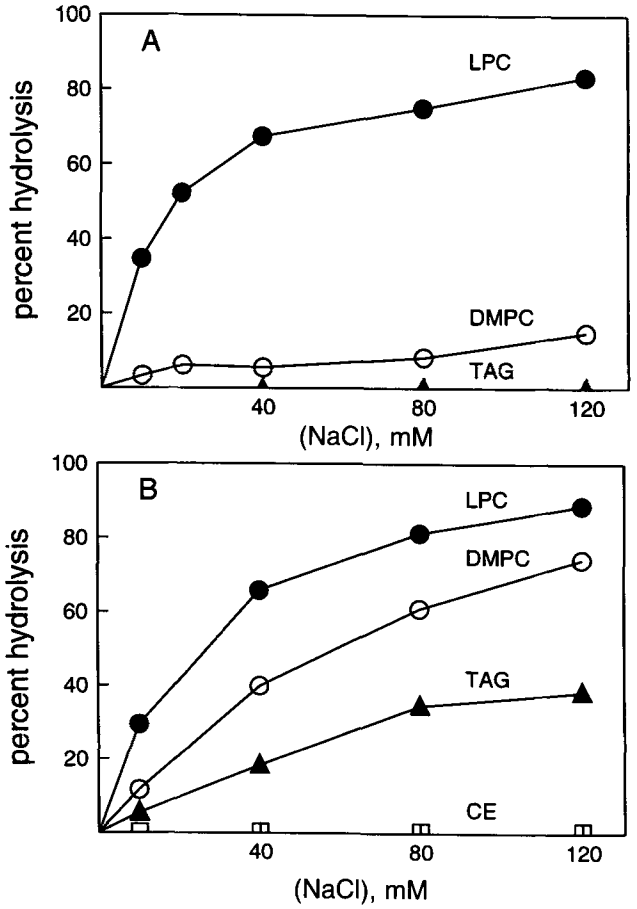

FIG. 2. Mixtures of 1-palmitoyl lysophosphatidylcholine (LPC) (●), dimyristoyl phosphatidylcholine (DMPC) (○), tripentadecanoylglycerol (TAG) (▲) and cholesteryl oleate (CE) (□) were incubated for 10 min with 0.5 mL of ethereal diazomethane and 0.1 mL of the indicated solutions of NaCl. Panel A, 0.1 mL methanol; Panel B, 0.3 mL methanol. Methyl esters were analyzed as described in the Materials and Methods section.

resulting fatty acids were methylated with diazomethane (data not shown).

DISCUSSION

The mechanism for the observed hydrolysis of lipid acyl groups by mixtures of diazomethane and aqueous inorganic salts is based on the observation by Kuhn and Ruelius (14), later reviewed by Huisgen (15), that diazomethane can methylate the anion of an inorganic salt, freeing the cation and thereby causing the aqueous solution to become basic (Equation 2):

$$CH_2N_2 + NaCl + HOH \rightarrow CH_3Cl + Na^+ + OH^- + N_2 \quad [2]$$

In the presence of methanol, this is sufficient to cause the formation of methoxide ion (Equation 3), a nucleophile that attacks the carbonyl carbon atom of the ester bond in a classic transesterification reaction (Equation 4):

$$CH_3OH + OH^- \rightarrow CH_3O^- + HOH \quad [3]$$

$$CH_3O^- + RO\text{–}CO\text{–}R' \rightarrow CH_3O\text{–}CO\text{–}R' + RO^- \quad [4]$$

It has been shown that methanolysis occurs faster than hydrolysis in methanol/water mixtures (16). Therefore, reaction in aqueous salt solutions, including plasma (10), will result in transesterification as described here. Higher salt concentrations probably stimulate the reaction through primary or secondary salt effects. Because free fatty acids are not esterified in basic medium by methoxide ions (17), it appears that in plasma the free fatty acids and the internal standard are methylated by diazomethane in the usual way and lipid acyl groups undergo methanolysis as outlined above, with the extent of reaction depending on specific conditions. When the Pace-Asciak procedure (10) was followed, the reaction occurred substantially faster with lysophosphatidylcholine than with other lipids, most likely because lysophosphatidylcholine partitions more readily into the aqueous/methanol phase.When sufficient methanol was present to effect a single phase, all lipids except cholesteryl esters were transesterified. In the two-phase system, triacylglycerols were not transesterified to any major extent, as was also reported by Lin *et al.* (11), but TAG participated readily in the reaction in single phase.

Trace amounts of unesterified fatty acids are found in serum, plasma, tissues and cells in the presence of overwhelming amounts of other lipids. Therefore a method for their analysis must be absolutely specific. Even a reaction of less than 1% of the bulk of the complex lipids would introduce significant errors. No matter how carefully conditions are controlled, use of a reagent such as methanol/acetyl chloride (13), that also transesterifies acyl groups (18), leads to overestimation of unesterified fatty acids accompanied by errors in their composition (19).

Some of the problems of nonspecificity of reagents such as methanol/acetyl chloride and methanol/HCl can be overcome by first isolating the unesterified fatty acids from the lipid extract, preferably by thin-layer chromatography (TLC). However, this is a time-consuming procedure and such small amounts of lipid are difficult to accurately localize on and to quantitatively isolate from a TLC plate. If the unesterified fatty acid fraction is contaminated with other trace lipids, such as diacylglycerols, there will be a corresponding overestimation due to methanolysis. Also, care must be taken to prevent oxidation of polyunsaturated fatty acids during chromatography. In contrast, methylation with diazomethane assures that the assay is specific for unesterified fatty acids. After methylation, small columns of silicic acid or commercial solid-phase extraction cartridges can be used to remove complex lipids that can cause problems if they are injected into the gas chromatograph.

Because of its somewhat basic character, diazomethane can also react with methanol to form methoxide, and can catalyze methanolysis of acetates in concentrated solutions at extended reaction times (48 h; Ref. 20). We have found no evidence that this reaction occurs at the concentrations and times employed for the methylation of fatty acids in lipid mixtures. Reaction of unsaturated fatty acids with ethereal diazomethane in the absence of methanol will lead to the generation of side products (21). Thus it

was shown that α,β-unsaturated fatty acids and α-keto acids, compounds unlikely to be encountered in usual lipid analyses, react with diazomethane not only at the carboxyl group but also at the double bond and the oxo group (22).

Our present data clearly show that diazomethane, when used as originally described (4), selectively methylates unesterified fatty acids and does not cause any transesterification of acyl groups. It is advisable to follow the procedure exactly, i.e., to use a reaction medium of diethyl ether/methanol (9:1, vol/vol) (4), or to carefully confirm that modifications to the method do not alter the results.

Although diazomethane is toxic and can explode under certain conditions, when in dilute solution and used in a functioning fume hood, it presents no greater hazard or inconvenience than do many other reagents that are in routine use in the laboratory. In our experience, diazomethane in diethyl ether/methanol solution is the only reagent that specifically methylates unesterified fatty acids and should therefore be used for the assay of free fatty acids in the presence of other lipids.

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COMMUNICATION

Ultrasound in Fatty Acid Chemistry: Synthesis of a 1-Pyrroline Fatty Acid Ester Isomer from Methyl Ricinoleate

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A novel 1-pyrroline fatty acid ester isomer [*viz.* 8-(5-hexyl-1-pyrrolin-2-yl)octanoate] has been synthesized from methyl ricinoleate by two routes with an overall yield of 42 and 30%, respectively. Most of the reactions are carried out under concomitant ultrasonic irradiation (20 KHz, *ca.* 53 watts/cm^2). Under such a reaction condition, the reaction time is considerably shortened, and product yields are high. Dehydrobromination under concomitant ultrasonic irradiation of methyl 9,10-dibromo-12-hydroxyoctadecanoate with KOH in EtOH furnishes methyl 12-hydroxy-9-octadecynoate (66%) within 15 min. Hydration of the latter under ultrasound with mercury(II)acetate in aqueous tetrahydrofuran yields exclusively methyl 12-hydroxy-9-oxo-octadecanoate (95%) in 30 min. The hydroxy group in the latter compound is transformed to the azido function *via* the mesylate, and treatment of the azido-oxo intermediate (methyl 12-azido-9-oxooctadecanoate) with Ph_3P under ultrasonic irradiation furnishes the requisite 1-pyrroline fatty acid ester (77%). The same azido-oxo intermediate has also been obtained by the oxidation of methyl 12-azido-9-*cis*-octadecenoate using benzoquinone and a catalytic amount of Pd(II)chloride in aqueous tetrahydrofuran under concomitant ultrasonic irradiation (90 min) to give the product in 45% yield. The latter reaction does not take place even under prolonged silent stirring of the reaction mixture.

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2,5-Dialkyl pyrrolines and pyrrolidines have been identified in the venom of the fire ant *(Solenopsis punctaticeps)* (1,2) and have been shown to possess necrotizing and hemolyzing properties (3,4). A number of synthetic procedures have been reported for the preparation of dialkyl pyrrolines with emphasis on the subsequent production of 2,5-disubstituted pyrrolidines for entomological studies (5–9).

We have reported earlier the synthesis and properties of a long-chain fatty ester derivative [methyl 8-(5-hexyl-2-pyrrolin-1-yl)octanoate, **1**] containing a 1-pyrroline nucleus in the alkyl chain from methyl *iso*-ricinoleate (methyl 9-hydroxy-12-*cis*-octadecenoate) (10). In the present paper we describe the transformation of the very readily available methyl ricinoleate (methyl 12-hydroxy-9-*cis*-octadecenoate) from castor oil to a novel 1-pyrroline isomer [methyl 8-(5-hexyl-1-pyrrolin-2-yl)octanoate, **2**] with the imine bond (C=N) of the pyrroline nucleus linked to the C-9 position instead of to C-12 (see Scheme 1). The requisite 1-pyrroline fatty ester isomer (**2**) was synthesized by two different approaches from methyl ricinoleate. Both approaches make use of ultrasound irradiation during the various synthetic steps to accelerate the formation of key intermediates. The synthetic routes are illustrated in Figure 1.

(1), R = $CH_3(CH_2)_5$; R' = $(CH_2)_7COOCH_3$

(2), R = $(CH_2)_7COOCH_3$; R' = $CH_3(CH_2)_5$

SCHEME 1

MATERIALS AND METHODS

Ricinoleic acid was obtained from castor oil by the partition method described by Gunstone (11). Petroleum ether refers to the fraction with b.p. 40–60°C. Thin-layer chromatographic (TLC) analysis was done on microscope glass plates coated with silica gel (type 60, silica gel GF_{254}, 0.1 mm layer; Merck, Darmstadt, Germany). Column chromatographic separation was achieved on silica gel (type 60, 70–230 mesh; Merck) by gradient elution using mixtures of petroleum ether/diethyl ether ranging from

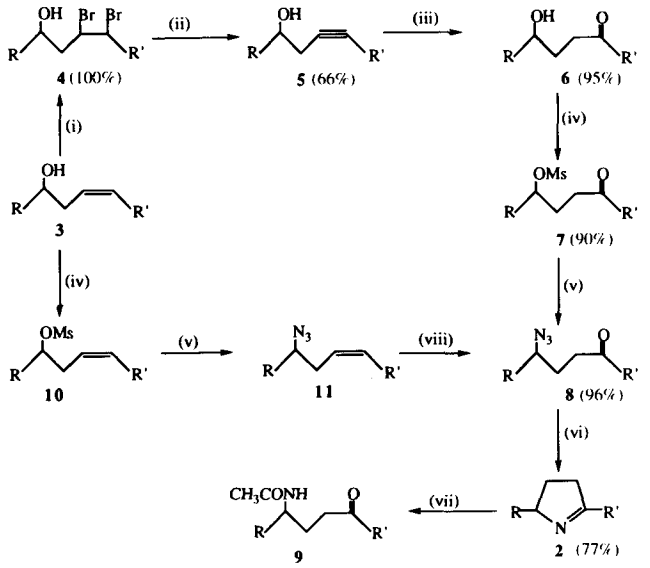

FIG. 1. Synthesis of 1-pyrroline fatty ester. Reagents and conditions: (i) Br_2, Et_2O, 20°C, 15 min; (ii) KOH, EtOH, ultrasound, 30 min; (iii) Hg $(OAc)_2$, tetrahydrofuran (THF), H_2O, ultrasound, 25 min; (iv) MsCl, Et_3N, CH_2Cl_2, –5°C, 30 min; (v) NaN_3, DMF, 80°C, 2 h; (vi) PPh_3, THF, ultrasound, 30 min; (vii) Ac_2O, 90°C, 1 h; (viii) $PdCl_2$, benzoquinone, THF, H_2O, ultrasound, 90 min.

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Abbreviations: ECL, equivalent chain length; GLC, gas–liquid chromatography; IR, infrared; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; R_f, retardation factor; THF, tetrahydrofuran; TLC, thin-layer chromatography.

1:0 to 2:3, vol/vol, depending on the polarity of the product. Gas–liquid chromatographic (GLC) analysis was carried out on a Hewlett-Packard model 5970 gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a microbore SE-30 (0.53 mm diameter, 2.65 μm film thickness, 10 m length) or SPB1 column (0.20 mm diameter, 0.20 μm film thickness, 30 m length) (Supelco, Bellefonte, PA) using nitrogen (20 mL/min) as the carrier gas at a column temperature of 190°C with the flame-ionization detector set at 210°C. External standards of methyl myristate, palmitate and stearate were used as reference compounds, and equivalent chain length (ECL) values were calculated for each component (12). Infrared (IR) spectra were obtained on liquid films on a Shimadzu model IR-470 photospectrometer (Shimadzu Corp., Kyoto, Japan). Nuclear magnetic resonance (NMR) spectra (in $CDCl_3$) were obtained on a JEOL FX90Q (90 MHz) or JEOL GSX-270 (270 MHz) instrument (JEOL Ltd., Tokyo, Japan). Ultrasonication was carried out using a 20 KHz (*ca.* 53 W/cm^2) ultrasound horn (Undatim Ultrasonic S.A., Louvain-la-Neuve, Belgium) with the reaction mixture contained in a water-jacketed cell. Low-resolution mass spectra were recorded using a Hewlett-Packard gas chromatograph fitted with a Mass Selective Detector™. High-resolution mass spectra were recorded on a Finnigan Mat 95 mass spectrometer (Finnegan Mat Corp., San Jose, CA).

Methyl 9,10-dibromo-12-hydroxyoctadecanoate (**4**). Bromine (5.6 g, 3.5 mmol) was added to a solution of methyl ricinoleate (**3,** 2.0 g, 6.4 mmol) in diethyl ether (30 mL) and stirred for 15 min. The reaction mixture was successively washed with aqueous sodium thiosulfate solution (5%, 10 mL), water (20 mL), and dried over anhydrous sodium sulfate. The ethereal mixture was filtered and the filtrate evaporated to give crude methyl 9,10-dibromo-12-hydroxyoctadecanoate (**4,** 3.0 g, 100%) as a viscous liquid (13,14). TLC, retardation factor (R_f) 0.6 (petroleum ether/diethyl ether, 7:3, vol/vol); IR (cm^{-1}): 3450 (*br*), 1740(*s*), 840(*m*), 760(*m*) and 720 (*s*); ^{1}H NMR ($CDCl_3$, δ): 0.88(*t*, 3H, C*H*$_3$), 1.2–1.8(*m*, 20H, C*H*$_2$), 1.9–2.1(*m*, 4H, C*H*$_2$), 2.2(*s*, 1H, CH-O*H*), 2.3(*t*, 2H, 2-*H*), 3.6(*s*, 3H, COOC*H*$_3$), 3.8(*m*, 1H, C*H*-OH), 4.6(*m*, 2H, C*H*-Br).

Methyl 12-hydroxy-9-octadecynoate (**5**). A mixture of compound **4** (3.0 g, 6.3 mmol), ethanol (95%, 30 mL) and KOH (5.3 g, 9.5 mmol) was sonicated for 30 min using a 20 KHz ultrasound horn at 20°C. The reaction mixture was acidified with HCl (6M, 20 mL) and extracted with diethyl ether (3 × 30 mL). The ethereal extract was washed with water (30 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was refluxed with borontrifluoride/methanol (14%, w/w, 5 mL) in absolute methanol (30 mL) for 10 min. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 50 mL). The solvent was evaporated, and silica column chromatographic purification of the residue using a mixture of petroleum ether and diethyl ether (85:15, vol/vol) as eluent gave pure methyl 12-hydroxy-9-octadecynoate (**5,** 1.3 g, 66%). TLC, R_f 0.5 (petroleum ether/diethyl ether, 7:3, vol/vol); IR (cm^{-1}): 3450 (*br*), 1740 (*s*), 1460 (*m*), 1435 (*m*), 1360 (*m*) and 1170(*m*). ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.6 (*m*, 20H, C*H*$_2$), 2.04 (*s*, 1H, CH-O*H*), 2.2 (*m*, 4H, 8-*H* and 11-*H*), 2.3 (*t*, 2H, 2-*H*), 3.66 (*s*, 3H, COOC*H*$_3$) and 3.69 (*m*, 1H, C*H*OH); ^{13}C NMR ($CDCl_3$, ppm): 14.1 (C-18), 18.7 (C-8), 22.6 (C-17), 24.9 (C-3), 25.2 (C-14), 27.7 (C-11), 28.6 (C-7), 28.9, 29.3, 31.8 (C-16), 33.9 (C-2), 36.2 (C-13), 51.4 (COO*C*H$_3$), 70.3 (C-12), 76.2 (C-10), 83.1 (C-9) and 174.3 (C-1).

Methyl 12-hydroxy-9-oxooctadecanoate (**6**). A mixture of compound **5** (2.0 g, 6.4 mmol), tetrahydrofuran (30 mL), water (30 mL) and mercury(II)acetate (4.1 g, 12.8 mmol) was sonicated for 25 min at 20°C. The reaction mixture was acidified with dilute HCl (2M, 20 mL) and extracted with diethyl ether (2 × 50 mL). The ethereal extract was washed with water (2 × 20 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated and silica column chromatography afforded pure methyl 12-hydroxy-9-oxo-octadecanoate (**6,** 2.0 g, 95%). TLC, R_f 0.4 (petroleum ether/diethyl ether, 7:3, vol/vol); IR (cm^{-1}): 3500 (*br*), 1740 (*s*), 1700 (*s*), 1460 (*m*), 1380 (*m*); ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.6 (*m*, 22H, C*H*$_2$), 2.10 (*s*, 1H, CH-O*H*), 2.30 (*t*, 2H, 2-*H*), 2.5 (*t*, 4H, 8-*H*, 10-*H*), 3.4–3.6 (*m*, 1H, C*H*-OH) and 3.6 (*s*, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, ppm): 14.03 (C-18), 22.59 (C-17), 23.83 (C-7), 24.86 (C-3), 25.67 (C-14), 28.98, 29.30, 30.98 (C-11), 31.85 (C-16), 34.07 (C-2), 37.80 (C-13), 39.10 (C-10), 42.89 (C-8), 51.40 (COO*C*H$_3$), 71.48 (C-12), 174.22 (C-1) and 212.00 (C-9).

Methyl 12-mesyloxy-9-oxooctadecanoate (**7**). A mixture of compound **6** (1.0 g, 3.0 mmol), methanesulfonyl chloride (0.8 g, 7.0 mmol), triethylamine (1.0 mL) and dichloromethane (10 mL) was stirred at 0–5°C for 30 min. The reaction mixture was diluted with dichloromethane (50 mL) and successively washed with dilute HCl (2M, 4 mL), water (20 mL), and dried over anhydrous sodium sulfate. The mixture was filtered and the filtrate evaporated to give crude methyl 12-mesyloxy-9-oxooctadecanoate (**7,** 1.1 g, 90%) as a viscous oil. TLC, R_f 0.4 (petroleum ether/diethyl ether, 1:1, vol/vol), IR (cm^{-1}): 2960, 2850, 1740, 1710, 1355, 1180, 970, 900, 760, 740 and 700; ^{1}H NMR (CDCl, δ): 0.9 (*t*, 3H, C*H*$_3$), 1.2–1.4 (*m*, 18H, C*H*$_2$), 2.0 (*m*, 4H, 11-*H*, 13-*H*), 2.3 (*t*, 2H, 2-*H*), 2.45 (*t*, 4H, 8-*H*, 10-*H*), 2.90 (*s*, 3H, C*H*$_3$ of OMs), 3.6 (*s*, 3H, COOC*H*$_3$) and 4.70 (*quintet*, 1H, C*H*-OMs); ^{13}C NMR ($CDCl_3$, ppm): 14.03 (C-18), 22.53 (C-17), 23.67 (C-7), 24.54 (C-14), 24.92 (C-3), 28.22, 29.03, 29.36, 29.68 (C-11), 31.74 (C-16), 34.02 (C-2), 34.86 (C-13), 37.74 (C-10), 38.62 (*C*H$_3$ of OMs), 42.74 (C-8), 51.30 (COO*C*H$_3$), 82.94 (C-12), 174.06 (C-1) and 209.65 (C-9).

Methyl 12-azido-9-oxooctadecanoate (**8**). A mixture of compound **7** (1.1 g, 2.7 mmol), sodium azide (0.4 g, 6 mmol) and dimethylformamide (10 mL) was stirred at 80°C for 2 h. Water (20 mL) was added, and the reaction mixture was extracted with diethyl ether (2 × 40 mL). The ethereal extract was washed with water (30 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated, and silica column chromatographic purification gave methyl 12-azido-9-oxooctadecanoate (**8,** 0.8 g, 96%). TLC, R_f 0.4 (petroleum ether/diethyl ether, 4:1, vol/vol); GLC, ECL 19.6 (SPB1), IR (cm^{-1}): 2930, 2857, 2098, 1741, 1714; ^{1}H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.5 (*m*, 14H, C*H*$_2$), 1.6–1.8 (*m*, 6H, C*H*$_2$), 1.9 (*m*, 2H, 11-*H*), 2.30 (*t*, 2H, 2-*H*), 2.41 (*t*, *J* = 7.0 Hz, 2H, 8-*H*), 2.54 (*t*, *J* = 7.0 Hz, 2H, 10-*H*), 3.25 (*m*, 1H, 12-*H*), 3.60 (*s*, 3H, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, ppm): 14.03 (C-18), 22.57 (C-17), 23.76 (C-7),

24.88 (C-3), 26.04 (C-14), 28.18, 28.94, 29.03, 29.08, 29.31 (C-11), 31.69 (C-16), 34.04 (C-2), 34.53 (C-13), 38.90 (C-10), 42.95 (C-8), 51.46 ($COOCH_3$), 62.36 (C-12), 174.25 (C-1) and 210.20 (C-9).

Compound **8** was also obtained from methyl 12-azido-9-*cis*-octadecenoate (**11**) as follows. A mixture of methyl 12-azido-9-*cis*-octadecenoate (**11**, 0.4 g, 1.2 mmol), benzoquinone (0.19 g), palladium(II)chloride (59% Pd content, 0.018 g), tetrahydrofuran (20 mL) and water (5 mL) was sonicated at 20°C for 90 min. Dilute HCl (2M, 50 mL) was added, and the reaction mixture was extracted with diethyl ether (2 × 40 mL). The ethereal extract was successively washed with dilute NaOH (0.5 M, 15 mL), water (30 mL), and brine (20 mL), and dried over sodium sulfate. The ethereal mixture was filtered, and the solvent of the filtrate was evaporated. The residue was purified by silica column chromatography to yield compound **8** (0.2 g, 45%).

Methyl 8-(5-hexyl-1-pyrrolin-2-yl)octanoate (**2**). A mixture of compound **8** (0.8 g, 2.3 mmol), triphenylphosphine (0.8 g, 3.0 mmol), and anhydrous tetrahydrofuran (20 mL) was sonicated at 80°C for 30 min. Water (40 mL) was added, and the reaction mixture was extracted with diethyl ether (2 × 30 mL). The ethereal extract was washed with water (30 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated, and silica column chromatographic purification of the residue using a mixture of petroleum ether and diethyl ether (2:3, vol/vol) as the eluent furnished the 1-pyrroline derivative **2** (0.5 g, 77%). TLC, R_f 0.5 (petroleum ether/diethyl ether, 3:2, vol/vol); GLC, ECL 18.6 (SPB1); IR (cm^1): 2926, 2854, 1741, 1644 (C=N, cyclic), 1463, 1197 and 1171; ^1H NMR ($CDCl_3$, δ): 0.89 (*t*, 3H, CH_3), 1.20–1.50 (*m*, 14H, CH_2), 1.50–1.80 (*m*, 6H, CH_2), 2.02 (*m*, 2H, ring CH_2, 11-*H*), 2.30 (*t*, 4H, 2-*H*, 8-*H*), 2.45 (*t*, *J* = 7.0 Hz, 2H, 10-*H*), 3.65 (*s*, 3H, $COOCH_3$) and 3.90 (*m*, *br*, 1H, N-C*H*, 12-*H*); ^{13}C NMR ($CDCl_3$, ppm): 14.09 (C-18), 22.64 (C-17), 24.92 (C-3), 26.65 (C-7, C-14), 28.66 (C-11), 29.04, 31.91 (C-16), 33.90 (C-8), 34.08 (C-2), 36.73 (C-13), 37.00 (C-10), 51.36 ($COOCH_3$), 72.59 (C-12), 174.17 (C-1) and 176.88 (C-9); mass spectrometry (MS) [*m/z* (relative intensity)]: M^+ 309.2664 (calc. 309.2667); 309 (4), 280 (19), 278 (25), 236 (14), 236 (14), 224 (12), 180 (56), 168 (12), 167 (100), 166 (14), 152 (11), 110 (13), 97 (12), 96 (31), 83 (27), 82 (66) and 55 (15).

Methyl 12-acetamido-9-oxooctadecanoate (**9**). A mixture of compound **2** (0.34 g, 1.1 mmol) and acetic anhydride (3.5 mL) was stirred at 90°C for 1 h. The reaction mixture was cooled and ice-water (15 mL) was added. The precipitate was collected by suction filtration, washed with cold water (5 × 10 mL) and recrystallized from a mixture of diethyl ether and ethyl acetate (1:3, vol/vol) to give compound **9**. TLC, R_f = 0.5 (hexane/diethyl ether/methanol, 1:3:1 by vol); ^1H NMR ($CDCl_3$, δ): 0.89 (*t*, 3H, CH_3), 1.20–1.50 (*m*, 14H, CH_2), 1.5–1.8 (*m*, 8H, CH_2), 1.95 (*s*, 3H, CH_3CONH), 2.30 (*t*, 2H, 2-*H*), 2.4 (*t*, *J* = 7.0 Hz, 2H, 8-*H*), 2.5 (*t*, *J* = 7.0 Hz, 2H, 10-*H*), 3.6 (*s*, 3H, $COOCH_3$), 3.9 (*m*, 1H, C*H*-$NHCOCH_3$) and 5.3 (*d*, *J* = 9.0 Hz, 1H, N*H*$COCH_3$); ^{13}C NMR $CDCl_3$, 14.07 (C-18), 22,58 (C-17), 23.5 ($NHCOCH_3$), 23.76 (C-7), 24.86 (C-3), 25.85 (C-14), 28.9 (C-11), 29.02, 31.76 (C-16), 34.04 (C-2), 35.81 (C-13), 39.59 (C-10), 42.95 (C-8), 49.48 (C-12), 51.48 ($COOCH_3$), 169.82 ($NHCOCH_3$), 174.28 (C-1) and 211.49 (C-9); MS [*m/z* (relative intensity)]: 69 (11), 99 (19), 114 (95), 128 (40), 156 (66), 157 (21), 168 (18), 170 (100), 185 (16), 242 (22), 266 (17), 278 (21), 284 (29), 296 (22), 310 (52), 326 (41), 338 (11), 339 (2), 369 (3).

RESULTS AND DISCUSSION

In our first reported synthesis of a 1-pyrroline fatty acid ester derivative, *iso*-ricinoleic acid (9-hydroxy-12-*cis*-octadecenoic acid, isolated from the seeds of *Wrightia tinctoria*) was used as the starting substrate (10). The presence of two methylene groups between the unsaturated center and the hydroxy function in the alkyl chain of this natural, unsaturated hydroxy fatty acid provided the suitable retrosynthetic steps for obtaining the target molecule (**1**) through a key intermediate (a 1,4-azido-oxo derivative). The 1-pyrroline fatty acid ester (**1**) synthesized was shown to have the C=N of the pyrroline nucleus attached to the C-12 of the alkyl chain of the substrate (*iso*-ricinoleic acid). As *W. tinctoria* seed oil is not readily available as compared to castor oil (a ready source of ricinoleic acid, 12-hydroxy-9-*cis*-octadecenoic acid), the focus of this project was therefore to obtain 1-pyrroline fatty acid derivatives from ricinoleic acid. The key intermediate in this synthesis plan was to obtain a similar 1,4-azido-oxo intermediate. Cyclization of the latter would therefore lead to the requisite 1-pyrroline fatty acid derivative. However, employing ricinoleic acid as the starting substrate, the position of the azido and oxo function are in the reversed order as compared with the 1,4-azido-oxo intermediate derived from *iso*-ricinoleic acid. Hence, the 1-pyrroline fatty acid product (**2**) is anticipated to have the C=N bond attached to C-9 of the alkyl chain of the fatty acid instead of the C-12.

Two synthetic routes leading to the target molecule (**2**) were envisaged. The first route involved the bromination of methyl ricinoleate (**3**) to give the corresponding dibromo derivative (**4**), which under concomitant ultrasound treatment (30 min) with KOH in ethanol furnished methyl 12-hydroxy-9-octadecynoate (**5**) in 66% yield. Hydration of compound **5** with mercury(II)acetate in aqueous tetrahydrofuran (THF) was also carried out under ultrasonic irradiation for 25 min to yield exclusively methyl 12-hydroxy-9-oxooctadecanoate (**6**) in almost quantitative yield (95%). The above debromination and hydration experiments normally required 6 h of reflux and 4 d of stirring at room temperature, respectively, for the reactions to be complete. Conversion of the hydroxy function in compound **6** to the azido group was achieved *via* the mesylate (**7**) to give the key intermediate **8** (methyl 12-azido-9-oxooctadecanoate). Subsequent cyclization according to the method described by Lambert *et al.* (15) of compound **8** with PPh_3 but under concomitant ultrasonic irradiation (30 min) furnished the requisite 1-pyrroline derivative (**2**) in 92% yield.

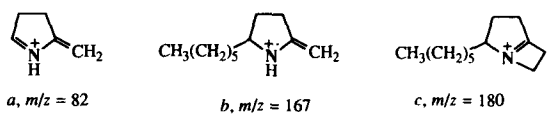
The second route leading to the target molecule (**2**) followed a similar approach as that reported for methyl 8-(5-hexyl-2-pyrrolin-1-yl)octanoate (compound **1**) from methyl *iso*-ricinoleate (10). Methyl ricinoleate was first transformed to the mesylate (**10**) and then converted to methyl 12-azido-9-*cis*-octadecenoate (**11**). The latter was then oxi-

dized with benzoquinone in the presence of palladium(II)chloride in aqueous THF under concomitant ultrasonic treatment (90 min) to yield exclusively the required key intermediate **8**. When this reaction was carried out by stirring at room temperature for 7 d, a very low yield of compound **8** (12%) was obtained. Increasing the temperature of the reaction caused large amounts of side products to be formed due to overoxidation. The use of ultrasound at room temperature provided not only the optimum condition for this chemical transformation, but also allowed for regiospecific oxidation. Reaction of compound **8** with Ph_3P gave the requisite 1-pyrroline derivative **2.** Both routes made use of ultrasound where reactions were expected to require long reaction times.

The final product (compound **2**) was characterized by a combination of physical methods, and the position of the imine (C=N) bond in the ring system was established by ring opening reaction of compound **2** to the acetamido derivative followed by mass spectral analysis.

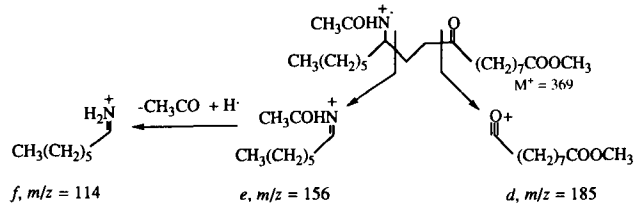
From the IR spectral analysis of compound **2,** the strong absorption bands at 1644 and 1741 cm^{-1} indicated the presence of the imine (C=N) and ester (C=O) function, respectively. The presence of the 1-pyrroline ring system was further supported by the signals at δ 2.02 and 2.45 for the methylene protons of the ring and the characteristically broad multiplet at δ 3.90 for the pyrroline ring CH proton adjacent to the nitrogen atom. Confirmation of the 1-pyrroline nucleus was further obtained from the ^{13}C NMR spectrum of compound **2**. The chemical shift of the imino (C=N) carbon atom of the 1-pyrroline system appeared at 176.88 (C-9) and the ring methine carbon atom at 72.59 (C-12) ppm. The C=N bond of the pyrroline system caused deshielding of the adjacent methylene carbon atoms with signals appearing at 33.90 and 36.73 for the C-8 and C-13 carbon atom, respectively. The shift of the remaining methylene carbon atom of the five-membered heterocyclic system appeared at 28.66 (C-11) ppm. The shielding γ-effect of the nitrogen atom of the pyrroline ring caused the shifts of the C-7 and C-14 carbon atoms to appear at 26.65 ppm.

The ^1H and ^{13}C NMR spectra of compound **2** were very similar to those obtained on compound **1** (10). The mass spectral analysis of compound **2** further confirmed the presence of the pyrroline nucleus from the peak at *m/z* 82 (Scheme 2, ion *a*, 66%), and the position of the pyrroline ring system between C-9 and C-12 was implied by the peaks at *m/z* 167 (*b*, 100) and 180 (*c*, 56). The only remaining doubt was the position of the imine bond. To determine



a, *m/z* = 82 *b*, *m/z* = 167 *c*, *m/z* = 180

SCHEME 2



f, *m/z* = 114 *e*, *m/z* = 156 *d*, *m/z* = 185

SCHEME 3

the position of the C=N linkage, compound **2** was converted to the corresponding acetamido-oxo derivative (**9**) by reaction with acetic anhydride. The mass spectral analysis of compound **9** confirmed the oxo function at the C-9 position from the peak at *m/z* 185 (Scheme 3, ion *d*, 16%). The linkage of the acetamido group was confirmed from the peak at *m/z* 156 (*e*, 66%) and the subsequent cleavage of the $CH_3C{\equiv}O^+$ moiety from this ion fragment to give the characteristic ion fragment at *m/z* 114 (*f*, 95%) (16). These results confirmed the linkage of the imine bond (C=N) at the C-9 carbon atom. In the case of compound **1,** the mass spectrum of the acetamido derivative did not give an ion fragment at *m/z* 114, as the acetamido group was linked to the C-9 carbon atom (10).

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Rapid Determination of Double Bond Configuration and Position Along the Hydrocarbon Chain in Cyclic Fatty Acid Monomers[1]

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This study reports the structural elucidation of diunsaturated 5- or 6-membered ring cyclic fatty acid monomers (CFAM) isolated from heated flaxseed oil by complementary gas chromatography (GC)–mass spectrometry (MS) and GC–matrix isolation–Fourier transform infrared spectroscopy (MI–FTIR). Infrared measurements of CFAM were carried out on methyl ester derivatives as well-resolved chromatograms were obtained on a polar 100% cyanopropyl polysiloxane capillary GC column. By contrast, electron ionization MS of methyl ester derivatives was of limited value because of double bond migration during the ionization process in the mass spectrometer. This communication reports definitive MS fragmentation patterns that can confirm ring position and double bond position along the fatty acid chain in 1,2-disubstituted CFAM determined as 2-alkenyl-4,4-dimethyloxazoline derivatives. Double bond configuration (*cis, trans,* or conjugated *cis,cis*) in CFAM was confirmed by GC–MI–FTIR. The presence of CFAM, degradation products found in used frying oils, is a potential source of dietary toxicity.

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Several decades ago, it was suspected that unsaturated cyclic fatty acid monomers (CFAM) are formed in frying oils. Consequently, several attempts were made to elucidate their structures by various analytical techniques (1–10). CFAM, which are reported to be potentially toxic (6,11–13) and are readily absorbed by the digestive system (14,15), were found at levels up to 0.5% in frying oils collected from restaurants in the United States (16).

Electron ionization (EI) mass spectrometry (MS) is of limited value in identifying unsaturation sites in CFAM methyl esters because in the EI mode double bonds tend to migrate along the aliphatic hydrocarbon chain (17,18). Hence, EI mass spectra of methyl esters cannot distinguish between closely related CFAM structures, and more suitable derivatives had to be found. Recent developments in the identification of CFAM isolated from seed oils (19) and heated sunflower oil (20) have been reported. The nature of the cyclic monoenoic fatty acids formed from linoleic acid in sunflower oil heated to 275°C was determined by gas chromatography–mass spectrometry (GC–MS) of the picolinyl ester derivatives following isolation by silver ion high-performance liquid chromatography (20). Compared to CFAM methyl ester derivatives, picolinyl ester derivatives inevitably suffer some loss of resolution during GC separation (20), and it is rarely possible to interpret mass spectra from mixtures satisfactorily (20). Although several derivatives have been proposed for locating double bonds (21), 2-alkenyl-4,4-dimethyloxazoline was selected because it has been successfully used to characterize unsaturated fatty acids with a terminal (monosubstituted) 5-membered ring (22). Moreover, in contrast to picolinyl ester derivatives, the resolution obtained in the GC separation of CFAM oxazoline derivatives was as good as that of methyl esters under identical GC conditions.

In the present study, a complex mixture of diunsaturated CFAM methyl esters was successfully converted to 2-alkenyl-4,4-dimethyloxazoline derivatives (21,22). The resulting CFAM oxazolines exhibited definitive EI mass spectra with distinctive fragmentations; thus the location of double bonds along the fatty acid chain of all components of the CFAM mixture could be determined. Matrix isolation–Fourier transform infrared spectroscopy (MI–FTIR) (23,24) offered equally unequivocal and complementary information on double bond configuration.

By using capillary GC–EIMS and GC–MI–FTIR, it was possible to rapidly establish the location of double bonds along the hydrocarbon chains in cyclic dienoic fatty acid monomers, the double bond configuration, the molecular weight, the location of the ring along the fatty acid chain, the ring size and the degree of unsaturation of diunsaturated C_{18} CFAM in complex mixtures isolated from heated flaxseed (linseed) oil.

MATERIALS AND METHODS

Materials were obtained from the following sources: refined linseed oil from Cargill (Riverside, ND); silica gel from Mallinckrodt (St. Louis, MO); urea from International Biotechnologies, Inc. (New Haven, CT); solvents (reagent grade) from Fisher (Pittsburgh, PA); Wilkinson's catalyst [tris(phenylphosphine)rhodium(I) chloride] from Strem Chemical Co. (Newburyport, MA); and deuterium from Alpha Products (Ward Hill, MA).

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Abbreviations: AMP, 2-amino-2-methyl-1-propanol; CFAM, cyclic fatty acid monomers; EI, electron ionization; FAME, fatty acid methyl esters; FTIR, Fourier transform infrared spectroscopy; GC, gas chromatography; MI, matrix isolation; MS, mass spectrometry; PFK, perfluorokerosene.

A test portion of oil was heated at 275°C under nitrogen for 12 h as previously described (25).

Refined linseed oil was saponified with potassium hydroxide, and the unsaponifiables were removed as detailed in the American Oil Chemists' Society official method Ca 6a 40 (26). The fatty acids recovered were converted to methyl esters as described previously (27). Published procedures (25) were followed for the separation of fatty acid methyl esters (FAME) from polar lipids by silicic acid column chromatography and for the isolation of CFAM methyl esters by urea fractionation of the nonpolar FAME fraction. The urea fractionation step was carried out twice, and the optimum ratio of urea to FAME was 3:1.

A portion of the isolated mixture of CFAM methyl esters was catalytically hydrogenated over platinum oxide by using a microhydrogenator (Supelco, Inc., Bellefonte, PA) as described earlier (9). Unsaturated FAME were deuterated by the method of Rakoff and Emken (28).

The method of Fay and Richly (21) for derivatizing FAME to oxazolines was modified as follows. About 150 μL 2-amino-2-methyl-1-propanol (AMP) was added to 24 mg of neat methyl esters in a 2-mL reaction vial. The vial was suspended in a wax bath held at 175°C for 6 h. The content of the vial was cooled and transferred with 5 mL methylene chloride to a 250-mL separatory funnel containing 40 mL petroleum ether. The funnel content was shaken, and the petroleum ether layer was washed with 40 mL deionized water and then dried with sodium sulfate. The solution was evaporated under a stream of argon, and the residue was dissolved in isooctane.

Low resolution GC–EIMS analyses were obtained using a Hewlett-Packard (Avondale, PA) 5890 series II gas chromatograph coupled to a Fissons VG (Wytheshawe, United Kingdom) Autospec Q mass spectrometer and OPUS 2000 data system. Version 1.6C software was used for the GC–MS system. The capillary GC column used (CP-Sil-88; Chrompack, Inc., Bridgewater, NJ), 50 m × 0.22 mm (i.d.), was coated with 0.19-μm stationary phase film. Adjusting the capillary GC column head pressure to 10 psi gave chromatographic profiles comparable to those obtained with a flame-ionization detector. GC–MS conditions were as follows: splitless injection with helium sweep restored 1 min after injection; injector and transfer lines held at 230°C; oven temperature program, 75°C for 2 min after injection, 20°C/min to 185°C, hold for 15 min, 4°C/min to 225°C, hold for 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/decade. Filament emission was 200 μA at 70 eV. Ion source temperature was 250°C.

Gas-chromatographic separations with MI–FTIR detection were performed on a Hewlett-Packard Model 5890 instrument. A Mattson Cryolect MI–FTIR system (Madison, WI) was used as detailed earlier (29).

RESULTS AND DISCUSSION

The total ion chromatogram obtained for the mixture of CFAM is shown in Figure 1. The oxazoline mass spectra confirmed the molecular weight of unsaturated C_{18} CFAM, the ring size, the degree of unsaturation, the position of the ring, and the location of double bonds along the chain. The CFAM oxazoline EI mass spectra consisted mainly of an even-mass homologous series in which the most intense peak in each cluster was at *m/z* 126 + 14*n* (*n* = 0, 1, 2, etc.). The pattern of peaks 14 mass units apart, occurring as a result of the cleavage of methylene groups (22), was interrupted when a double bond and/or a ring was present along the hydrocarbon chain. A double bond between carbons n and n+1 in a given hydrocarbon chain was indicated by an interruption of 12 mass units between fragments with n and n–1 carbon atoms (30). Rings caused large gaps of 68 (cyclopentyl), 82 (cyclohexyl), 66 (cyclopentenyl) or 80 (cyclohexenyl) mass units.

Typical examples of mass spectra for diunsaturated CFAM extracted from the heated flaxseed oil include two spectra of compounds that have a cyclopentenyl ring and either a *trans* double bond in the oxazoline 2-alkenyl substituent (Fig. 2A) or a *cis* double bond in the hydrocarbon (*n*-butene) substituent (Fig. 2B). The signal at *m/z* 113 arises from a McLafferty rearrangement involving the dimethyl oxazoline ring (30), whereas *m/z* 126 is reportedly formed *via* a cyclization displacement reaction induced by the nucleophilic center (30). The double bond configuration for these compounds was assigned on the basis of the position of infrared bands. Specifically, a cyclopentenyl

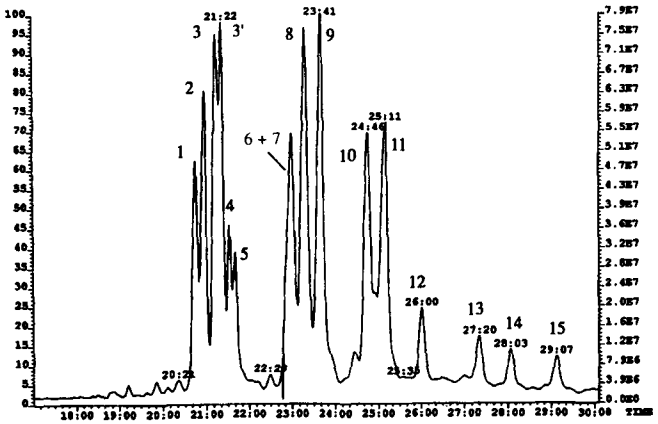
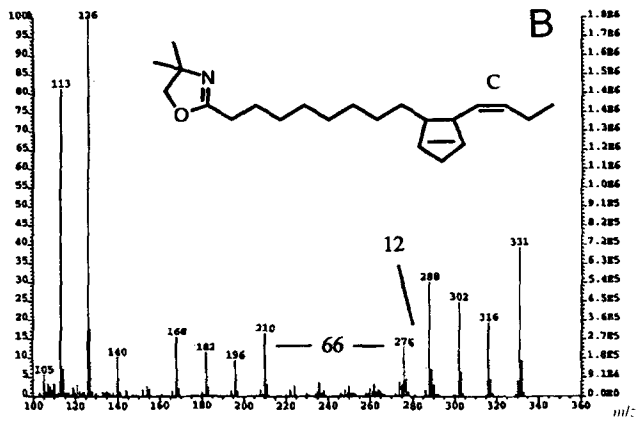
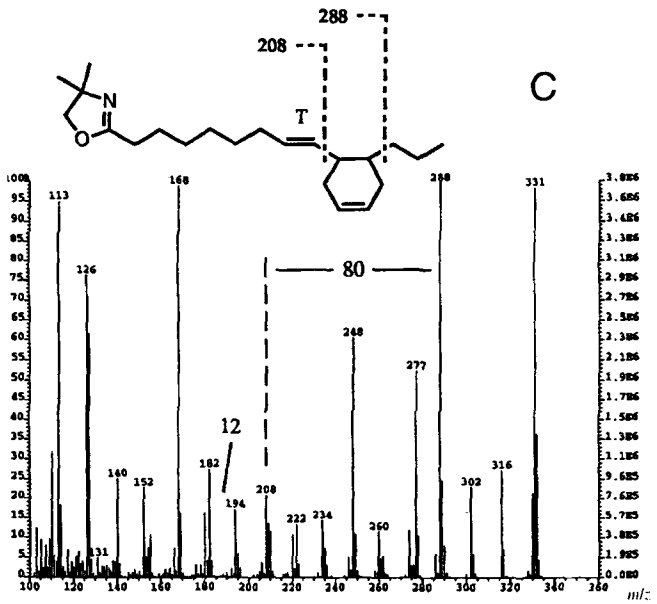


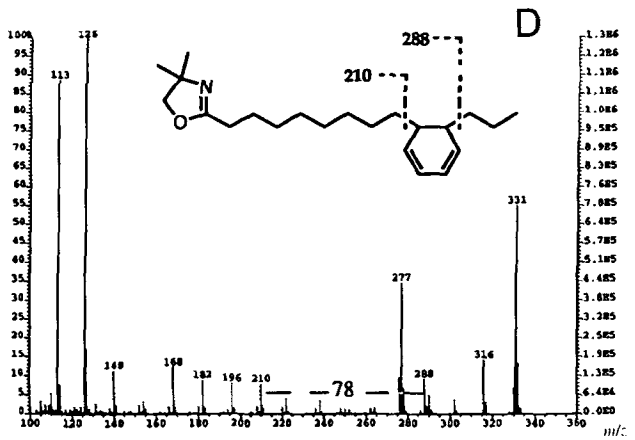
FIG. 1. Total ion chromatogram for the mixture of diunsaturated C_{18} cyclic fatty acid monomers (CFAM) oxazolines isolated from heated flaxseed (linseed) oil. CFAM having a cyclopentenyl ring (peaks 1–4, 6 and 7) eluted before those with a six-membered ring (peaks 8–13) on a 100% cyanopropyl polysiloxane capillary gas chromatography (GC) column. Peak 5 is due to the oxazoline derivative of linoleic acid. The structures that gave rise to peaks 14 and 15 are being investigated further. The identities of the alkenyl substituents at C-2 in 2-alkenyl-4,4-dimethyloxazolines that gave rise to the different GC peaks are as follows: 1, 8-(2-but-*trans*-1-enyl-cyclopentenyl)octyl; 2 and 4, 9-(2-propyl-cyclopentenyl)non-*trans*-8-enyl; 3, 9-(2-propyl-cyclopentenyl)non-*cis*-7-enyl; 3′ and 6, 8-(2-but-*cis*-1-enyl-cyclopentenyl)octyl; 7, 9-(2-propyl-cyclopentenyl)non-*cis*-8-enyl; 8 and 9, 8-(2-propyl-cyclohex-*cis*-4-enyl)oct-*trans*-7-enyl; 10 and 11, 8-(prop-*trans*-1-enyl-cyclohex-*cis*-4-enyl; 12 and 13, 8-(2-propyl-cyclohexa-*cis,cis*-3,5-dienyl)octyl. It is noted that the numbering of carbon atoms n and n+1 in the original fatty acid chain corresponds to carbons n–1 and n, respectively, along the 2-alkenyl chain of the oxazoline derivative.

ring *cis* double bond gave rise to bands near 3061 cm^{-1} (=C–H stretch) and 716 cm^{-1} (=C–H deformation) (8,31). A *trans* unsaturation site in a hydrocarbon chain produced weak features near 3035 and 3003 cm^{-1} (stretch) and a relatively strong band at 970 cm^{-1} (deformation) (32); a *cis* double bond in a chain produced a band of medium intensity near 3006 cm^{-1} (stretch) (32). The synthesis of standard C_{18} mono- or diunsaturated CFAM with a cyclohexenyl ring was reported (4,33,34), but CFAM with a cyclopentenyl ring (with two *cis* hydrogen atoms on the ring double bond) have yet to be prepared (10). The ring 1,2-disubstitution pattern was previously established for several cyclopentenyl (3,10) and cyclohexenyl (1,2,4,6,7) CFAM isolated from heated linseed oil by analyzing their oxidation products.

Mass spectra for a mono- and a diunsaturated six-membered ring CFAM are shown in Figures 2C and 2D, respectively. The odd-mass peak at *m/z* 277 (M-54), probably due to a retro Diels-Alder reaction, was not observed after hydrogenation (Fig. 2E). The compounds exhibited two sharp infrared bands of interest at 3032 (stretch) and 664 (deformation) cm^{-1} that are characteristic of a *cis* double bond in







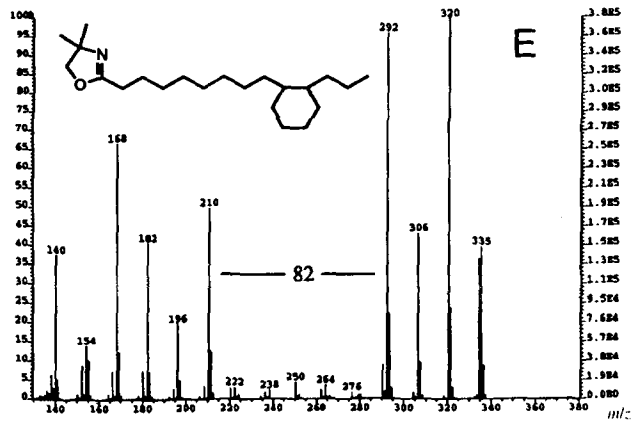


FIG. 2. A. Electron ionization (EI) mass spectrum for 2-[9-(2-propyl-cyclopentenyl)non-*trans*-8-enyl]-4,4-dimethyloxazoline indicating the location and degree of unsaturation in the ring and the position of the double bond in the chain. The location of the *cis* double bond in a 1,2-disubstituted five-membered ring could be either between C-3 and C-4, or between C-4 and C-5, B. EI mass spectrum for 2-[8-(2-but-*cis*-1-enyl-cyclopentenyl)octyl]-4,4-dimethyloxazoline. C. EI mass spectrum for 2-[8-(2-propyl-cyclohex-*cis*-4-enyl)oct-*trans*-7-enyl]-4,4-dimethyloxazoline. D. EI mass spectrum for 2-[8-(2-propyl-cyclohexa-*cis*,*cis*-3,5-dienyl)octyl]-4,4-dimethyloxazoline. Conjugation was deduced from Fourier transform infrared data (see text). E. EI mass spectrum for the saturated CFAM 2-[8-(2-propyl-cyclohexyl)octyl]-4,4-dimethyloxazoline. See Figure 1 for other abbreviation.

a six-membered ring (1,2,8); the latter species (Fig. 2D) gave rise to an additional feature at 723 cm^{-1} which indicated that two conjugated *cis* double bonds were present in the cyclohexadienyl ring. A similar pair of bands (near 655 and 740 cm^{-1}) was reported (31) for a 1,3-cyclohexadiene standard in the condensed phase at room temperature. It is noted that for a retro Diels-Alder reaction to occur (*m/z* 277) for this cyclohexadiene CFAM (Fig. 2D), isomerization of the ring double bonds would have to take place.

The combination of GC–EIMS and GC–MI–FTIR proved to be a powerful method for the rapid elucidation of the structure, and particularly for determining the double bond configuration and position along the fatty acid chain in mixtures of diunsaturated CFAM.

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CORRECTIONS

Tissue Phospholipid Fatty Acid Composition in Genetically Lean (Fa/-) or Obese (fa/fa) Zucker Female Rats on the Same Diet
Ph. Guesnet, J.-M. Bourre, M. Guerre-Millo, G. Pascal and G. Durand
Lipids 25, 517–522 (1990)

In this paper, in Table 4, the values for 20:3n-6 for kidney should read 1.0 ± 0.2 for lean rats and 1.3 ± 0.2[b] for obese rats (rather than 0.5 ± 0.2 and 0.3 ± 0.1), and for heart 0.3 ± 0.1 for lean rats and 0.7 ± 0.2[c] for obese rats (rather than 0.7 ± 0.2 and 0.5 ± 0.1).

In Table 6, the obese/lean ratios for brain for the Δ6-desaturase index should be 1.25 (and not 0.80), and for the Δ5-desaturase index 0.75 (and not 1.32).

Study on the Lipid Composition of Aging Fisher-344 Rat Lymphoid Cells: Effect of Long-Term Calorie Restriction
Serge Laganiere and Gabriel Fernandes
Lipids 26, 472–478 (1991)

In Table 2 of this paper, the value for 20:4 for long-term calorie restricted rats (LCR) at 12 months should be 17.98 ± 0.61 (rather than 7.98 ± 0.61). Similarly in Figure 3, upper right panel, the shaded LCR bar at 12 m for 20:4 should extend to 17.98 ± 0.61 (rather than to 7.98 ± 0.61).

Effects of *bis* Homoallylic and Homoallylic Hydroxyl Substitution on the Olefinic ^{13}C Resonance Shifts in Fatty Acid Methyl Esters
Philip E. Pfeffer, Philip E. Sonnet, Daniel P. Schwartz, Stanley F. Osman and David Weisleder
Lipids 27, 285–288 (1992)

Because of the recent unambiguous assignment of the C-9 and C-10 carbon-13 signals of *cis*- and *trans*-12-hydroxy-9-octadecenoic acids (*Nat. Prod. Lett. 3*, 65–69, 1993), the authors wish to make the following corrections:

In Table 3 (p. 287), column 5 (*cis* Ia) should be exchanged with column 6 (*cis* Ib), and column 7 (*trans* Ia) should be exchanged with column 8 (*trans* Ib).

On page 288, equation [3] should be exchanged with equation [4].

Reference 8 should read:

8. Lie Ken Jie, M.S.F., and Cheng, A.K.L. (1993) *Nat. Prod. Lett. 3*, 65–69.

Effects of Sodium Butyrate on the Transfer of Arachidonic Acid to Phosphatidylcholine in a Clonal Oligodendrocyte Cell Line (CB-II)
Synthia H. Sun, Kuo-Chi Chen and Yue-Wen Chen
Lipids 29, 467–474 (1994)

In this paper, in Table 2, the standard error for the last entry was inadvertently deleted. The value for total dpm in the presence of sodium butyrate should be 113201 ± 10825.

Thermal Adaptation Affects the Fatty Acid Composition of Plasma Phospholipids in Trout
Charlotte Wallaert and Patrick J. Babin
Lipids 29, 373–376 (1994)

In this paper, the first footnote to Table 1, line 9, should read "22°C-summer- and 8°C-winter-acclimated trout" (instead of "2°C-summer- and 8°C-winter-acclimated trout").

In addition Figures 1 and 2 were inadvertently transposed. The illustration at the right is Figure 1; the illustration at the left is Figure 2.

Editorial

When I decided to step down as Editor-in-Chief of *Lipids* by the end of this year, it was gratifying to know that I would leave the helm of a journal that is strong and healthy and that has earned the respect of the scientific community. The past ten years as *Lipids* Editor have been both challenging and rewarding. Looking back, I believe, we can be proud of what we accomplished.

To produce a quality scientific journal each month requires the special efforts of many, as well as cooperation and mutual trust. Authors, referees, editors, and production staff must work together and pool their efforts to assure success.

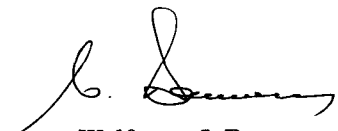
I wish to thank, first of all, our authors who have shown their confidence in our peer review and have submitted some of their most interesting work to our journal. Given an opportunity to further improve their papers after review, most authors have cooperated conscientiously and enthusiastically, and most were not even deterred by green ink.

It is with a deep sense of gratitude that I wish to specifically acknowledge the input and efforts of the many who served *Lipids* as referees over the years. A first-class journal is dependent on first-class reviewers. We have been fortunate, indeed, as we could count on the cooperation and advice of our many loyal referees, including some of the world's foremost experts in their respective fields. My special thanks to each and every one of them for volunteering their precious time and for letting us benefit from their knowledge and insights.

I also wish to express my appreciation to our Associate Editors for their tireless efforts and their help in keeping manuscripts moving along in the review process, and to the members of the *Lipids* Editorial Advisory Board for their encouragement and advice. I would also like to specifically thank my colleague and friend, Dr. Barbara Malewicz, who has served as Assistant to the Editor-in-Chief and as Index Editor. She kept the lines open to authors, referees and associate editors, and she did it efficiently and with grace. My cordial thanks to Barbara for her dedication, unparalleled hard work, encouragement, and support. She also kept our research program vigorous, thus allowing me to attend to my philanthropic editorial pursuits.

With the exception of the first volume of *Lipids*, which was edited by our Founding Editor, A. Richard Baldwin, all volumes of *Lipids* published since 1967 were edited at The Hormel Institute. With my stepping down as Editor-in-Chief, this 28-year tradition will come to an end. It is thus most appropriate to thank The Hormel Institute and its Board of Directors for making a home for *Lipids* throughout these years and for generously supporting its *Lipids* editors and the journal.

Looking back over the past ten years as *Lipids* Editor, I must say that I thoroughly enjoyed my daily excursions into the many areas of basic lipid science. I also very much cherished my personal contacts with authors, referees and associate editors from around the globe, many of whom I think of now as good friends. Serving as Editor-in-Chief of *Lipids* has been a special privilege, and it has been a most memorable and most rewarding experience. I am looking back with gratitude and pride. I trust I served the scientific community well.



Wolfgang J. Baumann
Editor-in-Chief